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REMARKS/ARGUMENTS

Claims 1-27 are pending. Claims 13, 14 and 27 have been withdrawn.

The amended first sentence of the specification properly claims priority to the relevant patent applications, thereby obviating the objection by the Examiner.

Applicants gratefully thank the Examiner for her courtesy in conducting an unscheduled telephonic interview on February 27, 2004, at which applicants' representative and the Examiner discussed, among other issues, that the instant claims are drawn to a *screening* method for determining a genotype associated with bipolar affective disorder, not to a genotype which is identified by such a screening method.

Rejections under 35 USC §112

The inventors of this application have identified six regions within the human genome that are associated with resistance to (protection from), or susceptibility to, familiar bipolar affective disorder (BPAD). In each of these regions, the inventors have identified at least one, and generally considerably more, markers that exhibit a highly significant linkage to resistance to, or susceptibility to, BPAD. These regions represent a relatively limited region (generally between about 10 and about 40 cM, as is discussed in more detail below) in which one can, *e.g.*, screen members of a family for these or for further markers that are correlated with BPAD. That is, they provide a region in which one can determine one or more markers (a genotype) that are linked, in a particular family of interest, to susceptibility to, or protection from, BPAD. Alternatively, the regions identified in the present application provide the basis for narrowing the size of the region that is linked to BPAD, or for strengthening the statistical correlations shown in this application. The above types of analyses can be accomplished by screening additional markers; by using the disclosed markers, *e.g.*, the markers recited in claims 5, 7, 8, 10 and/or 25; and/or by studying larger and/or different cohorts of subjects. The methods of the invention allow the formulation of sufficient detail such that determination of an allele(s) associated with increased resistance to BPAD may be determined (see, *e.g.*, page 5, lines 25-28 of the specification).

Thus, the instant claims are directed to screening methods, *e.g.*, a method for determining a genotype associated with increased resistance to (protection from) familial

bipolar affective disorder (BPAD) ... (independent claim 1); a method for determining the contribution of a chromosomal region to the presence of resistance to BPAD... (independent claim 23); or a method for determining a genotype associated with increased susceptibility to familial bipolar affective disorder (BPAD) ... (independent claim 24). In the method of claim 1, for example, a genotype of a (at least one) family member is determined; the BPAD status of the family member is determined; the genotype is compared with the BPAD status; and from this comparison, it is determined if the genotype is associated with increased resistance to BPAD.

The enablement and the written description rejections in the Office Action appear to be directed to claims that recite a genotype which is associated with increased or decreased resistance to bipolar disorder (*e.g.*, the *result* of a screening method for determining a genotype associated with increased or decreased resistance to BPAD). This is not what the instant claims recite. As discussed in the Reply filed on July 22, 2003, and again below, the specification provides both enablement and written description for performing the claimed screening methods.

With regard to the identification of the six regions, the Reply of July 22, 2003 reviewed in detail that at least one marker within each of the regions is significantly correlated with protection from or susceptibility to BPAD. For example, the following markers exhibit statistically significant linkage to susceptibility to BPAD: *D6S7* on chromosome 6; *D13S1* on chromosome 13; and *D15S45* on chromosome 15; and the following markers exhibit statistically significant linkage to resistance to (protection from) BPAD: *D4S2949* on chromosome arm 4p; *D4S397* on chromosome arm 4q; and *D11S133* and *D11 S29* on chromosome 11. That is, *D6S7*, *D13S1* and *D15S45* are each linked to susceptibility to BPAD with a SIBPAL p value of 0.0003 (see, *e.g.*, Figures 3, 4 and 5, respectively); *D4S2949* is linked to resistance to (protection from) BPAD with a SIBPAL p value of $< 5x10^{-5}$ (see, *e.g.*, Figure 6 and page 44, lines 11-13); *D4S2949* is linked to resistance to BPAD with a SIBPAL p value of <0.0001 (see, *e.g.*, Figure 7); and *D11S133* and *D11 S29* are linked to resistance to BPAD with a nominal p value of $< 5x10^{-5}$ (see, *e.g.*, page 44, lines 15-16). One of skill in the art would know that a SIBAL p value (statistical significance of the genetic linkage between markers based on sib-pair analysis) of < 0.001 is statistically significant under these circumstances. See also the specification at page 34, lines 13-15 ("those markers having a test statistic of p <0.001 in any one analysis type ... showed evidence for linkage"). The Examiner has presented no reasons or evidence to doubt the

assertion that these markers are highly correlated with BPAD.

The demonstration of these six strongly correlated markers would, in itself, be sufficient to identify six regions for further genotypic screening; each region extends for about 10cM on either side of one of those markers. It is well-known to those of skill in the art that one would reasonably expect that markers located at about 10cM (or, in some cases, more) from a marker of interest would be linked to that marker. For an example of current practice in the field, see the attached reference: Bosse *et al.* (2003) *Circulation* 107, 2361-2368. The Examiner's attention is drawn in particular to Figure 1, which shows linkage data for a number of markers. On Chr 2, the linked markers extend from about cM position 225 ± 20cM, for a span of about 40cM; on Chr 4, the linked markers extend from about cM position 45 ± 20cM, for a span of about 40cM; on Chr 14, the linked markers extend from about cM position 80 ± 10cM, for a span of about 20cM; and on Chr 17, the linked markers extend from about cM position 60 ± 10cM, for a span of about 20cM. Clearly, markers that extend for about 10cM (or more) on either side of a marker of interest are linked to that marker.

The present application goes beyond this initial identification of six regions of interest, by demonstrating numerous additional markers within each of the regions that exhibit strong correlation to BPAD. These data are shown both in the Figures and in the Tables in the specification. In general, the Figures present selected data points from the larger number of data points presented in the Tables. A summary of data identifying some of these additional markers is presented in Appendix A. In response to the request by the Examiner on page 4 of the Office Action, the Tables in which these data appear are explained in detail in the Appendix. Briefly, on **chromosome 6**, at least 4 markers have been identified within the ~38cM region between *6S344* and *D6S589* that are correlated with bipolar disease in a statistically significant manner. On **chromosome 13**, at least 4 markers have been identified within the claimed ~12cM region between *D13S218* and *D13S171* that are correlated with bipolar disease in a statistically significant manner. On **chromosome 15**, at least 5 markers have been identified within the claimed 20cM region between *D15S131* and *D15S117* that are correlated with bipolar disease in a statistically significant manner. On **chromosome arm 4p**, at least 19 markers have been identified within the 33.3cM region between *D4S431* and *D4S404* that are correlated with bipolar disease in a statistically significant manner. On **chromosome arm 4q**, at least 7 markers have been identified within the claimed 42.8cM region between *D4S402* and *D4S424* that are correlated with bipolar disease in a statistically significant manner. And on **chromosome 11**, at least 6 markers have been identified within

the claimed region between *D11S133* and *D11S29* that are correlated with bipolar disease in a statistically significant manner.

In the methods recited in the instant claims, members of a family of interest are tested for the presence of markers that lie within the indicated regions. As noted above, it would be evident to a skilled worker that markers within about 10 cM on either side of the highly correlated markers identified in the application are likely to be linked to those markers, thereby defining six linkage regions of interest. In fact the regions of interest are even more well characterized. For example, for chromosome 6, markers with statistical significance are spread across about 30cM; thus, the region of interest extends for at least this length. For chromosome 13, markers with statistical significance are spread across about 12cM; thus, the region of interest extends for at least this length. The length of the regions of interest for chromosomes or chromosome arms 15, 4p, and 4q extend for at least about 20cM, 33cM, or 45cM, respectively.

The identification of the six regions of interest is supported throughout the specification. The specification is replete with sophisticated analyses of the data, employing a variety of statistical techniques, which support the significance of the linkages. The fact that most of the data presented in the application have been published in prestigious peer review journals confirms the validity of these conclusions. Two such papers are attached for the convenience of the Examiner: Ginns *et al.* (1996) *Nature Genetics* 12, 431-435; and Ginns *et al.* (1998) *Proc Natl Acad Sci U S A* 95, 15531-15536. The allegation in the Office Action that other studies of linkage in BPAD may have been faulty (*e.g.*, as reported in the Berrettini reference cited by the Examiner, and as acknowledged in the specification, for example at page 2, line 27 to page 3, line 2) does not cast doubt on the accuracy or significance of the present findings.

The fact that several markers within a region of interest may not appear to be statistically significant by individual statistical methods does not cast doubt on the conclusion that the region is correlated with BAPD. For markers that lie within regions identified as showing significant linkage, but which by themselves do not exhibit such linkage, this result likely reflects the fact that these latter markers are less informative. This can result, *e.g.*, from less diverse alleles, *i.e.* fewer alleles. Furthermore, different statistical tests (modelings) produce different results. For instance, in linkage analyses, one can model recessive and/or dominant inheritance, while in allele sharing (SIBPAL, ASP, etc) the results are less dependent on *a priori* knowing the exact modes of inheritance. Since the genetics

(inheritance) working for each gene location was not known when this study was initiated, multiple statistical methods (*e.g.*, linkage, sibpal, ASP) were performed. These methods are described, *e.g.*, on pages 32-34 and 43-44 of the specification. As expected, different results were obtained with the different linkage analyses. The variability in values obtained by the different statistical methods is gene specific and varies from location to location. For example in Table 5, for marker D13S171, the Zmax dominant value of 0.000 appears highly significant, whereas the SIBPAL p value of 0.4905 might not appear by itself to be statistically significant. In a case like the above, when the nature of the inheritance was not known in advance, it is perfectly acceptable to identify the most significant values, and to conclude from them that the marker does, indeed, show linkage to BPAD. (The significance obtained under assumptions of a specific model can also be used to identify the genetic inheritance mechanisms.)

The Examiner appears to be concerned that the Blackwood reference cited in the Office Action (*Nature Genetics* 12, 427-430, 1996) identifies some markers in chromosome arm 4p as being associated with susceptibility to BPAD, whereas the present inventors identify markers in this region as being protective for BAPD. This is not a problem. It is not uncommon to find alleles within a given chromosome region, or even mutations within a given gene, that result in opposite phenotypic effects. For example, Geller *et al.* (2004) *Am J Hum Genet.* 74, 572-81 report that some mutations in the melanocortin-4 receptor gene predispose a subject to obesity, whereas other alleles in this gene have a negative association with obesity. This article is attached for the convenience of the Examiner. Furthermore, certain isoforms of ApoE (*e.g.*, ApoE 2) are protective for Alzheimer's Disease, whereas others (*e.g.*, ApoE 4) are associated with susceptibility to that disease. Three articles that discuss such results with the ApoE alleles are attached for the convenience of the Examiner: Sevush *et al.* (2000) *Am J Geriatr Psychiatry* 8, 254-256; Beyer *et al.* (2002) *NeuroReport* 13, 1403-1405; and Higgins *et al.* (1997) *Pharmacology Biochemistry and Behavior* 56, 675-685. The present inventors have recognized that markers on chromosome arm 4p correlate with *protection* from BPAD, an association that was not reported by Blackwood.

As for the allegation in the Office Action on page 7 that "The ability to screen for a wellness allele is even more unpredictable because it is very difficult to distinguish between the presence of a protective allele and the absence of a susceptibility allele," the specification clearly teaches how to distinguish between these two possibilities. For example, the specification states at page 39, lines 9-16 that

> Importantly, because of the long-term, longitudinal nature of the study, even the unaffected, mentally healthy individuals (those without any psychiatric illness) in these families have been closely followed, many for a period of years past the age of risk for BPAD. Consequently, rather than limit this genome-wide search to identifying susceptibility loci for the disease phenotype (BPAD), we tested the hypothesis that "protective" alleles may contribute to the absence of psychiatric illness (*i.e.*, mental health 'wellness') in unaffected family members in the 'high risk' pedigrees.

and at page 51, lines 13-16:

> Accordingly, an important step in our study which demonstrates that there are "protective" alleles was to show that there are "mentally healthy" individuals who share marker alleles that should increase the risk of developing BPAD, and yet, in the presence of 'protective' alleles these individuals do not manifest BPAD.

The existence of markers associated with wellness is well-established in the art. See, *e.g.*, the specification at page 54, line 16 to page 55, line 4. For example, wellness alleles have been reported for viral infections and Alzheimer's disease. Meyer *et al.* (1997) *AIDS* 11, F73-8 (attached) reports that a CCR-5 delta 32 mutant protects against HIV-1 disease progression. Beyer *et al.*, *supra* and Higgins *et al.*, *supra* disclose protective effects of ApoE2 alleles for Alzheimer's Disease. Again, the fact that the well respected journal, *Proc. Natl. Acad. Sci, USA*, published the above-mentioned paper by the present inventors, which shows that the markers on chromosome 4p from the present application are linked to a protective effect for BPAD, attests to the convincing nature of those data.

The specification is fully enabling for the recited screening methods: it teaches how to obtain samples from family members (*e.g.*, at page 19, lines 12-27 and Example I); how to perform the genotypic analysis (*e.g.*, in Example II); how to assess BPAD status (*e.g.*, in Examples I and IV) and how to analyze statistically whether the genotype determined is associated with increased or decreased resistance for BPAD (*e.g.*, in Example III). The specification teaches a variety of markers that can be used in the claimed methods, and teaches how additional markers can be generated (*e.g.*, at page 23, line 27 to page 27, line 10). The Office Action states at page 7 that "While one could conduct additional experimentation to determine whether markers exist within the recited regions on chromosome 4 and 11 and these newly discovered markers are associated with BPAD, the outcome of such research cannot be predicted." That is to some extent correct. The methods of the invention are screening methods, directed toward determining whether such markers

are, in fact, associated with BPAD.

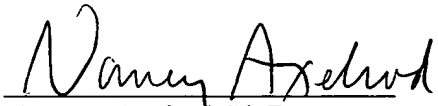
Not only is the specification enabling for the claimed invention, but it also provides written description. Contrary to the allegation of the Examiner on pages 12-14 of the Office Action, the specification provides even more than a "representative number" of markers within each of the claimed regions of the chromosomes. As discussed above, only one highly correlated marker is required to identify a region for further screening. In fact, the present application provides many more markers within each of the six regions identified. For example, some suitable markers to test for resistance to BPAD, flanking *D4S2949* (on chromosome arm 4p), are indicated at page 14, line 31 to page 15, line 4; some suitable markers flanking *D4S397* (on chromosome 4q) are indicated at page 15, lines 8-9; and some suitable markers on chromosome 11, in the ~20 cM span between *D11S133* and *D11S29*, are indicated at page 15, line 13. See also further candidate markers for resistance indicated on page 18, lines 5-13. Examples of suitable primers for identifying the above markers are indicated on page 22, line 1 to page 23, line 10. The specification also teaches some suitable markers to test for susceptibility for BPAD, and methods for identifying those markers.

In view of the preceding amendments and arguments, the application is believed to be in condition for allowance, which action is respectfully requested. If the Examiner would like to confer about the issues discussed herein, she is welcome to contact the undersigned applicants' representative.

Should any additional fee be deemed due, please charge such fee to our Deposit Account No. 22-0261 and advise us accordingly.

Respectfully submitted,

Date: **March 3, 2004**



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APPENDIX A

Table 5 (pages 35-36 of the specification; regions from chromosomes 6, 13 and 15), Table 7 (pages 46-47; region on chromosome arm 4p) and Table 8 (pages 47-48; region on chromosome arm 4q) present results of some of the linkage analyses presented in the above-referenced application.

In Table 5, several markers from each chromosome region are examined in four categories of subjects (which are defined in the footnote "Categories"). The fourth category contains the largest number of subjects, and therefore could provide more significant data than the other three groups. The data are presented for several statistical methods: linkage analysis (using either dominant ("dom") or recessive ("rec") models); SIBPAL p and ASP p. As discussed above, some markers appear to be significantly linked to BPAD in one, but not another type of statistical analysis. One of skill in the art would know that, if a given marker appears to be significantly linked to BPAD by one of the types of analysis, this is sufficient evidence that the marker is, in fact, linked.

Tables 7 and 8 present SIBPAL analysis of 4p and 4q markers, in either a single pedigree (110) or in a total of four pedigrees (110, 210, 310, 410). Nominal and simulated values are presented for each marker.

Some of the significant linkages are summarized below:

On **chromosome 6**, at least 4 markers have been identified within the ~38 cM region between *6S344* and *D6S589* that are correlated with bipolar disease in a statistically significant manner. The data are shown in Table 5:

D6S344, zmax/dom = 0.000 for each of the 4 patient groups studied;

D6S7, zmax/rec = 0.0003 for the first patient group; SIBPAL p= 0.0000, 0.0003 or 0.0001 for three of the patient groups;

D6S28, zmax/rec = 0.003, 0.000, 0.000, or 0.000 for the 4 patient groups;

D6S89, zmax/rec = 0.001 or 0.000 for two of the patient groups.

On **chromosome 13**, at least 4 markers have been identified within the claimed ~12 cM region between *D13S218* and *D13S171* that are correlated with bipolar disease in a statistically significant manner. The data are shown in Table 5 and/or in Figure 4:

D13S218, Table: SIBPAL p=0.0171 or 0.0175 for two of the patient groups;

D13DS1m, Fig. 4, SIBPAL p=0.0003;

D13DS1t, Fig. 4, SIBPAL p=0.0003;

D13S171, Table: Zmax/dom=0.000 for all 4 patient groups.

In addition, *D13S221*, which is outside the region that is currently claimed, is shown in Table 5 to have values of zmax/rec = 0.012 and Zmax/dom=0.000.

On **chromosome 15**, at least 5 markers have been identified within the claimed ~20cM region between *D15S131* and *D15S117* that are correlated with bipolar disease in a statistically significant manner. The data are shown in Table 5 and/or Fig. 5:

D15S36, Fig. 5 and Table 5, SIBPAL p=0.0114 or 0.0118 for two of the patient groups, Table 5, zmax/dom=0.000 for the 4th patient group;

D15S45, Fig. 5 and Table 5, SIBPAL p=0.0003 or 0.0018 for two of the patient groups;

D15S148, Fig. 5 and Table 5, SIBPAL p=0.0217, 0.0219, 0.0123 or 0.0360 for the 4 patient groups;

135yc, Fig. 5, SIBPAL p=0.102

D15S117, Table 5, zmax/dom=0.000 for the 4th patient group.

On **chromosome arm 4p**, at least 19 markers have been identified within the ~33.3cM region between *D4S431* and *D4S404* that are correlated with bipolar disease in a statistically significant manner. These data are shown in Table 7. In a few cases, significant values for markers are also shown Fig. 6:

Marker	Fig. 6 (SIBPAL p)	Table 7 (SIBPAL p, nominal, pedigrees 110-410)
D4S431		0.0110
D4S2366	0.0002	0.0002
D4S3007	0.0015	0.0014 (pedigree 110)
D4S394	0.0035	0.0007
D4S2983		$<1x10^{-4}$
D4S2923		0.0003
D4S615		$<1x10^{-4}$
Afman180xa9		$<1x10^{-4}$
D4S2928		$<5x10^{-3}$
D4S1582		0.0032
D4S107		$<5x10^{-5}$
D4S3009		0.0001
D4S2906		0.0004
D4S2949	0.0000	$<1x10^{-7}$
D4S1582	0.0005	

D4S2942		$<1x10^{-4}$
D4S3048		0.0036
D4S419	0.0002	0.0004
D4S404	0.0004	0.0004

Also, D4S391, which lies outside of the claimed region, is shown in Table 7 to have a SIBPAL p=0.0001 (pedigree 110).

On **chromosome arm 4q**, at least 7 markers have been identified within the claimed ~42.8 cM region between *D4S402* and *D4S424* that are correlated with bipolar disease in a statistically significant manner. The data are shown in Table 8 and/or in Figure 7:

Marker	Fig. 7 (SIBPAL p)	Table 8, (SIBPAL p, nominal, pedigree 110)
D4S402	<0.0001	
D4S409	0.0001	
D4S422		0.0108
D4S175	<0.0001	0.0471
D4S1579		0.0015
D4S397	<0.0001	$3x10^{-7}$
D4S424	0.0003	0.0481

On **chromosome 11**, at least 6 markers have been identified within the claimed region between *D11S133* and *D11S29* that are correlated with bipolar disease in a statistically significant manner. The six markers are *D11S394, D11S133, D11S147, CD3D, D11S285* and *D11S29*. The specification teaches on page 15, lines 10-13 that these six markers are significant, and indicates explicitly that *D11S133* and *D11S29* are linked to resistance to BPAD with a nominal p value of $< 5x10^{-5}$ (see, *e.g.*, page 44, lines 15-16). The Examiner has presented no reason to doubt the statement by applicants that all 6 markers exhibit statistically significant correlation.

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Genetics

A genome-wide search for chromosomal loci linked to mental health wellness in relatives at high risk for bipolar affective disorder among the Old Order Amish

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Communicated by Seymour S. Kety, National Institute of Mental Health, Bethesda, MD, October 19, 1998 (received for review May 12, 1998)

ABSTRACT Bipolar affective disorder (BPAD; manic-depressive illness) is characterized by episodes of mania and/or hypomania interspersed with periods of depression. Compelling evidence supports a significant genetic component in the susceptibility to develop BPAD. To date, however, linkage studies have attempted only to identify chromosomal loci that cause or increase the risk of developing BPAD. To determine whether there could be protective alleles that prevent or reduce the risk of developing BPAD, similar to what is observed in other genetic disorders, we used mental health wellness (absence of any psychiatric disorder) as the phenotype in our genome-wide linkage scan of several large multigeneration Old Order Amish pedigrees exhibiting an extremely high incidence of BPAD. We have found strong evidence for a locus on chromosome 4p at *D4S2949* (maximum GENEHUNTER-PLUS nonparametric linkage score = 4.05, $P = 5.22 \times 10^{-4}$; SIBPAL $P_{empirical}$ value $< 3 \times 10^{-5}$) and suggestive evidence for a locus on chromosome 4q at *D4S397* (maximum GENEHUNTER-PLUS nonparametric linkage score = 3.29, $P = 2.57 \times 10^{-3}$; SIBPAL $P_{empirical}$ value $< 1 \times 10^{-3}$) that are linked to mental health wellness. These findings are consistent with the hypothesis that certain alleles could prevent or modify the clinical manifestations of BPAD and perhaps other related affective disorders.

Bipolar affective disorder (BPAD) afflicts approximately 1% of the population and is associated with a high risk of suicide (1). Twin, family, and adoption studies have provided strong evidence for an important genetic component in the susceptibility to develop BPAD (2), but unlike for other common medical illnesses, robust biological markers have not been identified for BPAD, and genetic linkage studies have had to rely on categorical diagnoses. Genetic heterogeneity, phenocopies, genotyping errors, and the complexities of performing and interpreting statistical analyses may contribute to some of the inconsistences observed in these linkage studies (3–12). However, because the inheritance of BPAD is probably multifactorial, the possible involvement of multiple genetic components of small effect and/or the occurrence of major allelic effects only in epistasis must be considered. We previously have suggested (13), that in addition to susceptibility alleles, there could be alleles that reduce the risk of developing BPAD in a manner similar to that reported for other complex genetic disorders. If model-based linkage analyses are used, a false-negative linkage finding could result when individuals inherit disease susceptibility alleles but do not manifest the phenotype because of the presence of protective alleles. The inclusion of individuals who inherit susceptibility alleles but do not manifest disease because of protective alleles, or of individuals who inherit protective alleles but nevertheless manifest the disease, also will reduce the power of model-free (allele-sharing) analyses. Thus, regardless of whether model-based or model-free analyses are used, wellness or protective alleles could have a significant impact on linkage analyses.

Ascertainment of psychiatric disorders and health among several large multigenerational Old Order Amish pedigrees covers a period of more than 20 years. Throughout this longitudinal study, procedures for assessing and diagnosing subjects have remained constant and have included a thorough evaluation of all bipolar I (BPI) probands and their relatives (14–16). Morbid risk analyses have demonstrated a high prevalence of affective disorder among first-degree relatives of bipolar probands in these families (17). Because of the long-term, longitudinal nature of the study, the unaffected, mentally healthy individuals in these families also were followed, many for a period of years past the age of risk for BPAD. Consequently, rather than limit our genome-wide search to identifying susceptibility loci for the disease phenotype (BPAD), we tested the hypothesis that protective alleles may contribute to the absence of psychiatric illness (i.e., mental health wellness) in unaffected family members in these high-risk pedigrees. Because the mode of inheritance of any gene(s) modifying the relative risk for affective disorder is unknown (2) we relied exclusively on model-free linkage analyses. We now report strong evidence for linkage of DNA markers on chromosome 4p to mental health wellness in relatives at high risk for, but who did not develop, major affective disorder in several large multigenerational Old Order Amish pedigrees with an extremely high incidence of BPAD.

Abbreviations: BPAD, bipolar affective disorder; NPL, nonparametric linkage; BPI, bipolar I; GH-PLUS, GENEHUNTER-PLUS; IBD, identical by descent.

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§P.St.J. and R.A.P. contributed equally to this work.

MATERIALS AND METHODS

Diagnostic Assessment. Our genetic-epidemiologic study of BPAD among the Old Order Amish in southeastern Pennsylvania has been described in detail (18), including the methods for ascertainment and diagnostic documentation with informed consent (15, 19). Diagnoses were made, by using strict research diagnostic criteria (16), by a five-member psychiatric review board whose members were blind to pedigree membership, diagnostic opinions, treatment data from abstracted medical records, and genetic marker status. Twenty-five nuclear families were developed based on one or more confirmed cases of BPI, which formed the structure of pedigrees 110, 210, and 310. Our present report uses all of these earlier subjects plus additional expansions, especially in pedigree 410, so that our overall study now contains 346 samples, including those from 50 BPI individuals. The unaffected individuals (mentally well or healthy) are those for whom all SADS-L (schedule for affective disorders and schizophrenia-lifetime version) interview responses were negative, and no contradictory reports were given by family informants. Any individuals for whom some symptomatology was identified, even if it did not meet criteria for a formal diagnosis, were labeled as unknowns in our linkage analyses.

The method used for this longitudinal study is ethnographic and culturally appropriate to the field setting. Several members of each nuclear family with a BPI proband (BPI nuclear family) are seen annually. Those diagnosed with BPI or other major affective disorder undergo a yearly course-of-illness update. Parents of each BPI patient are visited regularly and have proven to be accurate informants. At least one unaffected sibling (control sample) of the married BPI patients has been interviewed yearly since 1990 in connection with a prospective study of children at risk for BPAD. Hence, at least three members and occasionally all members of each BPI nuclear family have been evaluated yearly. Individuals are interviewed anew with the complete SADS-L schedule whenever any abnormal mental or emotional symptoms are identified by the follow-up mechanisms. The long-term, systematic follow-up of the families in our study has demonstrated that onset of illness in the Old Order Amish usually is reported by multiple informants.

Patient Samples. Blood samples were generally collected after each first-degree relative (including parents, siblings, and children older than age 15) of the BPI probands had been interviewed with the complete SADS-L schedule. Samples were obtained with written informed consent and coded to maintain confidentiality. The phlebotomist was kept blind to pedigree relationships and diagnostic status. Lymphoblastoid cell lines, on an average of eight members of each nuclear family, were established at the Coriell Institute for Medical Research, Camden, N.J. and/or the Clinical Neuroscience Branch, Intramural Research Program, National Institute of Mental Health, Bethesda, MD. The NIGMS Human Genetic Mutant Cell Repository catalogue (20) contains updated pedigree and diagnostic information for several of the Amish pedigrees used in our study.

Genotyping. Genomic DNA was obtained from peripheral blood samples and/or immortalized lymphoblastoid cell lines as described (9). The order of typed markers on our mapping panels was obtained from the genetic location database (21).

DNA panels for PCR were in a 96-microtiter plate format, and aliquoted by using a BioMek robot (Beckman Instruments). The DNA samples were PCR-amplified separately with each of the DNA markers and then the PCR products were multiplexed, six markers per lane, for electrophoresis using GENESCAN software on ABI 373 instruments (Applied Biosystems Division, Perkin-Elmer). The genotypes subsequently were analyzed with GENOTYPER. DNA from several individuals was represented multiple times in the genotyping panels as controls to evaluate the consistency of genotypes across different gels.

Genetic Analysis Software (38) was used to identify problematic marker data, and a utility written in SPSS (SPSS, Chicago) generated a list of samples that needed to be rerun because of inheritance discrepancies or unreadable signals. We maximize the useful information by repeating the genotyping/analysis cycles until all possible DNA marker genotypes are obtained. Once genotyping for a marker was finished, the data were reanalyzed with G.A.S., and observed allelic mutations and other noninheritances were zeroed out in the data file and noted. Histograms indicating the marker allele size bins were generated. FASTLINK (22) was used to reanalyze the data before further statistical analyses.

Linkage Analyses. Model-free linkage analyses were conducted by using the two-point affected sib pair analysis program S.A.G.E. SIBPAL (23) and the multipoint analysis program GENEHUNTER-PLUS (GH-PLUS) (24). Because there were a few sibships with incomplete marker information, marker allele frequencies were estimated from the entire Old Order Amish family data set by using a maximum likelihood method implemented in the program MENDEL/USERM13 (25, 26). SIBPAL was used to identify markers showing an excess of alleles shared identical by descent (IBD) among unaffected, mentally healthy sib pairs. Under the null hypothesis of no linkage between a trait and marker, sib pairs would be expected to share on the average 50% of alleles IBD, but when a trait and marker are linked, IBD sharing will be increased in both affected and unaffected sibpairs. Because SIBPAL assumes marker allele frequencies appropriate for random samples, it underestimates the proportion of alleles shared IBD by concordant sib pairs when there is linkage. Multipoint analyses by using the model-free linkage program GH-PLUS produced nonparametric linkage (NPL) scores along points at the chromosomal region of interest. Two scoring functions are available in GH-PLUS: IBD sharing can be assessed among concordant relative pairs (NPL_{pairs}) or it may be assessed among larger groups of concordant relatives (NPL_{all}). Our analyses were conducted by using the NPL_{all} statistics as Kruglyak and colleagues (24) have demonstrated that the NPL_{all} statistic results in a more powerful test than the NPL_{pairs} statistic.

RESULTS

First, we analyzed our genome-wide scan dataset looking for evidence of chromosomal loci linked to mental health wellness (the absence of any psychiatric illness). In these analyses, only mental health wellness, in individuals who were over 45 years of age and had a first-degree BPI sibling in their family (pedigrees 110, 210, 310, and 410), was the linkage phenotype of interest (concordantly unaffected pairs) by using SIBPAL. Of more than 980 DNA markers, only five markers representing three chromosome regions had test statistics that were sufficiently outlying and that were likely to represent significant linkage results. Of the markers on chromosome 4p, *D4S2949*, which is located in the vicinity of the BPAD susceptibility locus reported by Blackwood *et al.* (11), had an empirical SIBPAL P value $<3 \times 10^{-5}$ (nominal P value $<1 \times 10^{-7}$). The marker *D4S397* on chromosome 4q had an empirical SIBPAL P value $= 9 \times 10^{-4}$ (nominal P value $= 3 \times 10^{-7}$) On chromosome 11q, two DNA markers (*D11S133* and *D11S29*), located over an approximately 20-cM region, each had a nominal P value $<5 \times 10^{-5}$ (SIBPAL; simulations were not performed). To supplement standard criteria for assessing the significance of our linkage analysis results, we used graphical techniques (Fig. 1) and the empirical assessment of P values (27–29). If each marker assessed in a pairwise linkage analysis is unlinked to the trait, then the P values associated with those markers should be uniformly distributed. In addition, the test statistics used to generate these P values (for instance, t tests in the case of SIBPAL) should follow an appropriate distribution. A plot (generated by using PROC CHART, SAS) of the test statistics obtained from each pairwise linkage analysis is shown in Fig. 1. The plot in the inset depicts a line that should be linear if all markers are unlinked. As seen in Fig. 1, there are outlying test statistic values that likely represent false null hypotheses, that is, evidence for significant linkage results. In addition, in the inset to

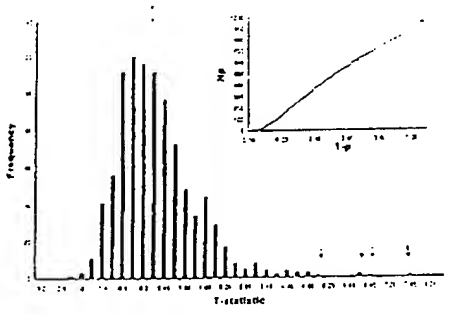

FIG. 1. Plot of test statistics obtained from the pair-wise linkage results. (*Inset*) A cumulative plot of *P*-values whose linearity would reflect uniformity in *P*-values associated with multiple linkage results whose null hypotheses were all true (see text). The outlying test statistics and *P*-values (denoted by arrows) were associated with markers, *D4S107* ($t = 6.24$), *D4S2949* ($t = 7.79$), *D4S2928* ($t = 5.03$), *D11S133* ($t = 6.09$), and *D11S29* ($t = 6.32$).

Fig. 1, the small upturned portion of the *P* value plot near values of 1 -- $P = 1$ represents departures from uniformity and hence most likely reflects false null hypotheses. Because of the effort required to investigate the significance of these findings and the previous evidence for a BPAD-related locus on chromosome 4 (11), we chose to examine DNA markers on chromosome 4 first for linkage to mental health wellness.

To evaluate the findings on chromosomes 4p and 4q in more detail, we genotyped the subpedigrees and nuclear families containing at least one sibling with BPI (Table 1) by using additional DNA markers in these interesting regions. Compared with our previous report (9) a larger number of individuals was included in these analyses (Table 1). In this report, model-free linkage analyses using SIBPAL and GH-PLUS (24) were performed by using mental health wellness as the linkage phenotype (Tables 2 and 3). In our analyses, individuals having a psychiatric diagnosis other than BPI, as well as those having psychiatric symptoms but no diagnosis, were classified in the unknown category for affected status. In the Amish Study sample of BPI patients ($n = 50$) the mean and median ages of onset (research diagnostic criteria) are 24 and 22 years, respectively. Hence, in all analyses we used a conservative age cutoff of 45 years to define family members with the unaffected wellness phenotype. We also

Table 1. Old Order Amish subjects included in linkage analysis

Subjects	Analysis categories	
	Mentally healthy	Unknowns
Pedigrees 110, 210, 310, 410		
≥25 years old	138	85
≥35 years old	109	114
≥45 years old	74	149
≥55 years old	52	171
Pedigree 110 only		
≥25 years old	45	32
≥35 years old	37	40
≥45 years old	31	46
≥55 years old	23	54

The category of unknowns includes individuals of unknown phenotype, individuals with psychiatric diagnoses other than BPI, and individuals who are mentally healthy but are younger than the particular age cut-off used in analyses. BPI individuals are not included in the unknown phenotype category. In pedigrees 110, 210, 310, and 410, 39 people were diagnosed with BPI, eight with BPII, 21 with recurrent depressive disorder, two with unipolar depressive disorder, and 15 with other psychiatric illness. In pedigree 110 alone, 18 people were diagnosed with BPI, two with BPII, 10 with major depressive disorder, and five with other psychiatric illness. Note: the individuals used in these linkage analyses represent only a subset of the entire Amish bipolar pedigrees because only nuclear families and subpedigrees containing a sibling with BPI were included.

examined the influence of different age cutoffs for defining well individuals and the contribution of different subpedigrees (families from pedigrees 110, 210, 310, and 410 versus only families from pedigree 110) on the test statistics for linkage. Well individuals younger than the specified age cutoff were considered to have an unknown affected status in the analyses.

On chromosome 4p, the maximum multipoint NPL values (GH-PLUS; including only individuals > age 45 years) were 4.05 ($P = 5.22 \times 10^{-4}$) for pedigree 110 and 4.05 ($P = 1.84 \times 10^{-4}$) for all pedigrees, respectively. The maximum multipoint NPL values (GH-PLUS; including individuals > age 45 years) for markers on chromosome 4q were 3.29 ($P = 2.57 \times 10^{-3}$) for pedigree 110 and 2.82 ($P = 4.43 \times 10^{-3}$) for all pedigrees, respectively. The GH-PLUS $-\log_{10} P$ value as a function of the map position at these locations on chromosome 4 is shown in Fig. 2. SIBPAL test statistics for markers on chromosomes 4p and 4q are shown in Tables 2 and 3. On chromosome 4 the lowest (nominal) *P* values obtained from the SIBPAL test statistics were for markers *D4S2949* (4p; $P < 1 \times 10^{-7}$) and *D4S397* (4q; $P = 3 \times 10^{-7}$). The maximum multipoint NPL values (GH-PLUS; including individuals > age 45 years) for markers on chromosome 11q were 2.43 for pedigree 110 and 2.49 for all pedigrees, respectively.

To obtain empirical *P*-values, we simulated genotype data by randomly assigning marker alleles to the founders and then assigning alleles to their descendants following Mendelian inheritance. Allowing for consanguineous matings, the entire family structure was used in marker assignment, thus taking into account all relationships between individuals in the dataset. For each simulation, after marker assignment, the pedigrees were trimmed down to that of the nuclear families used in the linkage analysis. SIBPAL then was run on the trimmed dataset, and test statistics for concordant and discordant sib pairs were obtained. The true *P* value is simply estimated as the proportion of replicates in which the simulated statistic is greater than or equal to the observed statistic, i.e., the probability that the observed result or something more extreme would be obtained by chance alone. Simulations were conducted for markers on chromosomes 4p and 4q. For each marker, 100,000 replicates were obtained. The empirical *P* value on chromosome 4p clearly meets the proposed criteria of significance for linkage (30).

DISCUSSION

If alleles exist that are associated with mental health wellness, then the identification of chromosome regions containing these alleles would be enhanced by studying the genetically at risk, mentally healthy members of large, multigenerational pedigrees like our Old Order Amish families. However, in trying to identify protective or wellness alleles, one must recognize that there are phenocopies that need to be considered in our analyses. Despite the extremely high risk for developing disease, some individuals are undoubtedly well because they do not inherit any (or all) of the requisite susceptibility alleles for BPAD. Because the age of greatest liability for onset of BPAD in the Old Order Amish is from early teens through 24 years of age, the misspecification of the well phenotype for individuals who eventually will develop BPAD would be greatest through this age period. In these Old Order Amish families susceptibility alleles for BPAD probably occur in very high frequency. An important step in demonstrating that there are protective alleles will be to show that there are mentally healthy individuals who share marker alleles that should increase the risk of developing BPAD, and yet, in the presence of protective alleles, these individuals do not manifest BPAD. For many of the markers, $\hat{\Pi}$, an underestimate of the proportion of alleles shared IBD in well sibpairs, increases with increasing age, i.e., a more stringent definition of the well phenotype. For example, with respect to marker *D4S2949* on 4p, $\hat{\Pi}$ is 0.60, 0.65 and 0.71 for age cutoff points of 25, 35, and 45 years, respectively. This finding suggests that increasing the age for inclusion eliminates some age-related well phenocopies.

Table 2. Results of SIBPAL analysis of 4p markers

Marker	Pedigree 110			Pedigrees 110, 210, 310, 410		
		P-value			*P*-value	
	$\hat{\Pi}$ (s.e.)	Nominal	Simulated	$\hat{\Pi}$ (s.e.)	Nominal	Simulated
D4S412	.4749 (.0621)	.6555	np	.5116 (.0539)	.4154	np
D4S431	.5734 (.0441)	.0523	np	.5921 (.0388)	.0110	np
D4S2366	.6781 (.0452)	.0002	.0005	.6024 (.0356)	.0027	.0094
D4S2935	.5066 (.0218)	.3825	np	.4998 (.0198)	.5043	np
D4S3007	.6233 (.0386)	.0014	.0023	.5632 (.0337)	.0330	.0496
D4S394	.6782 (.0513)	.0007	.0012	.5955 (.0421)	.0135	.0249
D4S2983	.7219 (.0484)	$<1 \times 10^{-4}$	np	.6090 (.0377)	.0025	np
D4S2923	.6661 (.0446)	.0003	np	.5902 (.0307)	.0022	np
D4S615	.7161 (.0393)	$<1 \times 10^{-4}$	np	.6223 (.0324)	.0002	np
Afm184za9	.7396 (.0446)	$<1 \times 10^{-4}$	np	.6220 (.0370)	.0008	np
D4S2928	.7333 (.0257)	$<5 \times 10^{-5}$	np	.6369 (.0272)	$<5 \times 10^{-5}$	np
D4S1605	.5453 (.0258)	.0440	.0472	.5795 (.0244)	.0011	0.0058
D4S1582	.6787 (.0616)	.0032	.0112	.6269 (.0557)	.0139	.0510
D4S107	.6557 (.0246)	$<5 \times 10^{-5}$	.0029	.6514 (.0243)	$<5 \times 10^{-5}$	.0088
D4S3009	.7325 (.0552)	.0001	np	.6237 (.0379)	.0008	np
D4S2906	.6460 (.0396)	.0004	np	.5853 (.0327)	.0055	np
D4S2949	.7077 (.0202)	$<1 \times 10^{-7}$	$<3 \times 10^{-5}$	.6888 (.0243)	$<1 \times 10^{-7}$	$<3 \times 10^{-5}$
Afm087zg5	.5229 (.0368)	.2686	np	.5114 (.0246)	.3218	np
D4S2944	.5647 (.0263)	.0093	np	.5428 (.0255)	.0483	np
D4S403	.6032 (.0492)	.0217	.0233	.5989 (.0443)	.0232	.0350
D4S2942	.7196 (.0308)	$<1 \times 10^{-4}$	np	.6627 (.0243)	$<1 \times 10^{-4}$	np
D4S2984	.5510 (.0396)	.1032	np	.5493 (.0297)	.0505	np
D4S1602	.6001 (.0561)	.0412	np	.5703 (.0383)	.0356	np
D4S1511	.6242 (.0489)	.0077	np	.5779 (.0315)	.0079	np
D4S2311	.7429 (.0279)	$<5 \times 10^{-5}$	np	.6327 (.0336)	.0001	np
D4S3048	.6628 (.0573)	.0036	np	.5998 (.0403)	.0078	np
D4S419	.5981 (.0270)	.0004	.0010	.5772 (.0319)	.0100	.0201
D4S404	.6785 (.0489)	.0004	.0010	.6428 (.0470)	.0020	.0072
D4S391	.7008 (.0487)	.0001	.0003	.6585 (.0470)	.0008	.0035

$\hat{\Pi}$ is the estimated proportion of alleles shared identical by descent. np, simulations not performed.

It is conceivable that virtually all cases of affective disorder in these families are caused by a common set of susceptibility alleles. The wellness or protective loci that we tentatively have identified could harbor alleles that prevent the manifestation of a BPAD spectrum phenotype, which also could include major depressive disorder. In our analyses the strongest evidence for protective alleles comes from pedigree 110, suggesting that such alleles may be more likely in this branch of the family. However, highly significant test statistics and multipoint logarithm of odds scores (using GH-PLUS) also are observed when pedigrees 110, 210, 310, and 410 are used for analyses (Fig. 2 *A* and *B*). The decreased sharing in proportion of alleles IBD for discordant pairs provides further support for the existence of alleles associated with the absence of affective disorder (mental health wellness) in these families (data not shown). In addition, epistatic interactions between alleles also could prevent or delay onset of an illness such as major depressive disorder from developing into BPAD. Indeed, as we increase the age-of-risk cutoff for defining the well phenotype from 25 to 45 years in our linkage analyses, the number of mentally healthy members decreases as expected, yet the evidence for linkage increases (data not shown).

There is some debate on the analysis of sibling pairs as to whether the use of inbred sibling pairs results in an increased number of false-positives if allele-sharing-based statistical methods are used (31). However, the arguments that (*i*) inbred sibling pairs are likely to share more genes than noninbred sibling pairs (i.e., have a kinship factor greater than 0.5) and (*ii*) that greater regions of the genome would show significant deviations from the expected noninbred sibling sharing value of 0.5 are incorrect when one is merely considering an analysis of sibling pairs involving only the transmission of alleles from parents to offspring. The transmission of alleles from parents to offspring will follow Mendelian ratios, and thus the null values for 0, 1, or 2 IBD sibling allele sharing in any population will be 0.25, 0.50, and 0.25, whenever only parental and sibling genotype information is used. However, if the origin of the parental alleles is taken into consideration, then there will be greater information about alleles shared by sibling pairs from inbred populations. For example, this increased information has the potential to resolve ambiguities in the sharing of alleles transmitted from homozygous parents, because the two copies of the allele in an inbred homozygous parent could be IBD. This information also could help resolve alleles shared by siblings identical in state into alleles shared IBD, showing that alleles transmitted to two offspring from different parents may be copies of the same allele because of the relatedness of the parents. If genealogy is taken into account, then the increased ability to resolve ambiguities in allele sharing would result in greater power in the analysis of inbred sibling pairs (31).

Ultimately, if inbreeding exists in a population from which sibling pairs have been gathered, but one ignores genealogical information by merely studying the transmission of alleles from parents to offspring, then no increase in false-positive linkage results will occur, because Mendel's law applies to inbred as well as outbred parent-offspring allele transmission studies. On the contrary, a decrease in power may result from inbred sibling pair analyses because spouses may manifest greater homozygosity and therefore provide less informative genotypes for parent-offspring-based linkage studies.

Genetic mapping of complex disorders with multifactorial inheritance could be especially difficult if, in addition to susceptibility alleles, individuals inherit protective alleles that prevent or reduce the risk of manifesting the disease phenotype. Even though model-based linkage analyses that do not allow for a multifactorial component are of only limited usefulness in these circumstances, they are still used frequently. In these instances, a false-negative linkage finding (type 2 error) could result when

Table 3. Results of SIBPAL analysis of 4q markers

	Pedigree 110			Pedigrees 110, 210, 310, 410		
		P-value			*P*-value	
Marker	$\hat{\Pi}$ (s.e.)	*Nominal*	Simulated	$\hat{\Pi}$ (s.e.)	Nominal	Simulated
D4S2303	.4616 (.0423)	.8145	np	.4670 (.0305)	.8585	np
D4S2985	.5754 (.0255)	.0027	np	.5403 (.0139)	.0025	np
D4S2423	.5445 (.0415)	.1453	.1373	.5445 (.0304)	.0743	.0846
D4S2286	.5533 (.0522)	.1570	np	.5225 (.0381)	.2780	np
D4S2959	.5035 (.0359)	.4619	np	.4906 (.0268)	.6370	np
D4S175	.5960 (.0558)	.0471	.0636	.5995 (.0484)	.0231	.0348
D4S422	.6198 (.0500)	.0108	np	.5685 (.0386)	.0403	np
D4S1576	.5290 (.0509)	.2861	np	.5377 (.0367)	.1545	np
D4S2294	.4960 (.0446)	.5351	np	.4867 (.0381)	.6358	np
D4S1579	.6206 (.0381)	.0015	np	.5740 (.0298)	.0077	np
D4S397	.7511 (.0449)	3×10^{-7}	.0009	.6586 (.0376)	5×10^{-6}	.0002
D4S3089	.4544 (.0348)	.9013	np	.4768 (.0261)	.8120	np
D4S2965	.5296 (.0581)	.3068	np	.5267 (.0366)	.2340	np
D4S192	.5135 (.0408)	.3715	np	.5040 (.0337)	.4525	np
D4S420	.5595 (.0539)	.1384	np	.5462 (.0389)	.1200	np
D4S1644	.5224 (.0521)	.3351	.2870	.5503 (.0362)	.0845	.0925
D4S3334	.5491 (.0254)	.0304	.0497	.5258 (.0287)	.1858	.1769
D4S1565	.5091 (.0373)	.4042	np	.5040 (.0271)	.4420	np
D4S1625	.5433 (.0454)	.1730	np	.5533 (.0339)	.0603	np
D4S424	.5901 (.0527)	.0481	np	.5950 (.0461)	.0226	np
D4S1604	.5501 (.0473)	.1480	np	.5095 (.0345)	.3919	np
D4S1548	.5597 (.0356)	.0511	np	.5814 (.0267)	.0016	np

$\hat{\Pi}$ is the estimated proportion of alleles shared identical by descent. np, simulations not performed.

individuals inherit disease susceptibility alleles but do not manifest the phenotype because of the simultaneous presence of protective alleles. If model-based methods are used, it is important to provide a reasonably low estimate of penetrance and include a multifactorial component in the model.

In the initial stages of analyzing a disorder like BPAD, which most likely displays multifactorial inheritance, robust model-free (allele sharing) methods are usually more useful than model-based linkage analysis (32). Concordant individuals should demonstrate excess allele sharing, even with the occurrence of phenocopies, genetic heterogeneity, high frequency of susceptibility alleles, and incomplete penetrance. Individuals who inherit susceptibility alleles but do not manifest disease because of protective alleles and individuals who inherit protective alleles but nevertheless manifest the disease will reduce the power of these analyses. Thus, regardless of the type of linkage analysis performed, the presence of protective alleles could have a major impact on identifying susceptibility loci.

Although the idea that protective alleles could modify (or even prevent) a behavioral phenotype like BPAD is relatively novel, there are examples where such protective alleles can affect the expression or inheritance of other Mendelian and multifactorial disorders. The severity of sickle cell anemia is influenced by genes that increase the amount of circulating fetal hemoglobin (33). Similarly, the genotype of the chemokine receptor CCR5 dramatically influences the kinetics of HIV-1 infection, where most individuals who are homozygous for a 32-bp deletion in the CCR5 gene encoding the coreceptor for macrophage-tropic HIV-1 are protected from virus infection (34). In Alzheimer's disease, apolipoprotein (Apo) E2, in contrast to ApoE4, appears to reduce the risk of developing the disease and may protect individuals who inherit a disease-associated ApoE4 allele (35). In an extended Italian family, Apo A-I$_{MILANO}$ protects against the development of both clinical and pathologic signs of atherosclerosis, despite significantly elevated plasma triglycerides and a markedly decreased level of high density lipoprotein-cholesterol (36). In the nonobese diabetic mouse model of human autoimmune insulin-dependent diabetes mellitus, partial protection from disease is provided by resistance alleles occurring singly at either the Idd3 or Idd10 non-major histocompatibility complex loci, whereas epistatic interaction between resistance alleles at these two loci produces nearly complete protection from diabetes (37).

There are several mechanisms by which wellness or protective alleles could affect the clinical manifestations of BPAD in the Old Order Amish. One possibility is that dominant acting protective alleles, either singly or acting together in epistasis, could prevent or modify the BPAD phenotype. The variable penetrance of illness or its heterogeneous clinical manifestations could result from resistance or protective alleles that alone provide only partial protection, while together with other genes produce epistatic interactions, resulting in a greater degree of modification of the phenotype. Alternatively, there also could be cellular target molecules, e.g., mood effectors, having forms that are either resistant or susceptible to the genetic and/or environmental susceptibility factors for BPAD. Individuals having resistant mood effectors would be protected from the effects of susceptibility alleles and/or environmental factors that result in the BPAD phenotype. In contrast, individuals with sensitive forms of these mood effectors would be vulnerable to developing the BPI phenotype when requisite BPAD susceptibility alleles and/or environmental factors are present. If epistatic interactions are required for manifestation of the effects of either susceptibility or protective alleles, the existence of resistant and sensitive forms of cellular effectors or protective alleles would be most apparent in families (or populations) where there is a high density of affected individuals such as the Old Order Amish in the present study. Regardless of the mechanism, the presence of wellness or protective alleles would have a significant impact on linkage analyses as evidenced by preventing the appearance of the BPAD phenotype (or its presentation as a forme fruste) in individuals who are otherwise genetically predisposed to developing illness.

Thus, we suggest that the identification of chromosomal loci harboring genes that contribute to the clinical manifestations of BPAD will likely require a multilocus approach that considers both additive and subtractive influences of alleles on the BPAD phenotype. The involvement of protective or wellness alleles in determining the manifestation of the BPAD phenotype would provide an attractive, testable explanation for at least some of the difficulty encountered in searches for BPAD susceptibility alleles.

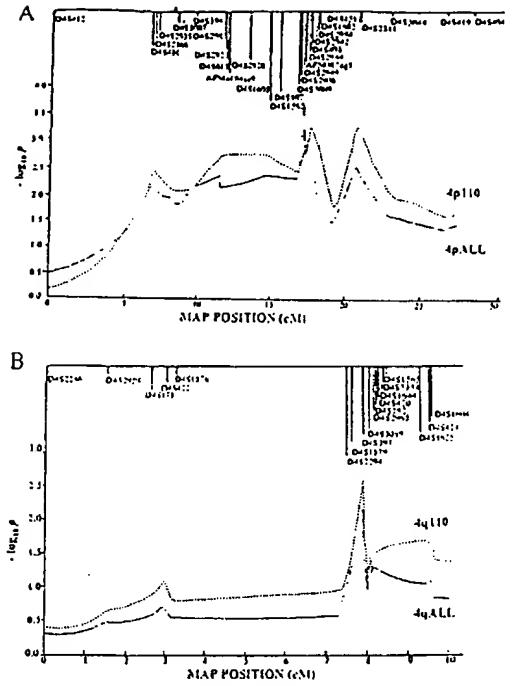

FIG. 2. Model-free linkage analysis of wellness by using GH-PLUS. Map position is in Kosambi centimorgans. The $-\log_{10} P$ was calculated by using P values generated by GH-PLUS on the assumption that the NPL score is standard normally distributed. A $-\log_{10} P$ of 4.0 corresponds asymptotically to a logarithm of odds score of 3.0. Only mentally healthy individuals 45 years of age or older were classified as being well (see text). (*A*) $-\log_{10} P$ for markers on chromosome 4p: dotted line, pedigree 110 only; and solid line, pedigrees 110, 210, 310, and 410. (*B*) $-\log_{10} P$ for markers on chromosome 4q: dotted line, pedigree 110 only, and solid line, pedigrees 110, 210, 310, and 410.

The test statistics from our analyses for alleles linked to the absence of psychiatric illness in the Old Order Amish are at least as significant as those reported for any susceptibility locus, and further investigations into the significance of these findings in the inheritance of BPAD are warranted. The identification and characterization of protective alleles and their gene products could lead to the development of a more rational and direct approach to effective therapy for affective disorders.

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A genome-wide search for chromosomal loci linked to bipolar affective disorder in the Old Order Amish

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The most characteristic features of bipolar affective disorder (manic-depressive illness) are episodes of mania (bipolar I, BPI) or hypomania (bipolar II, BPII) interspersed with periods of depression. Manic-depressive illness afflicts about one percent of the population, and if untreated, is associated with an approximately 20% risk of suicide[1]. Twin, family and adoption studies provide compelling evidence for a partial genetic aetiology, but the mode(s) of inheritance has not been identified[2]. Nonetheless, the majority of genetic linkage studies have assumed classical mendelian inheritance attributable to a single major gene. Although segregation analyses have yielded inconsistent results (with most studies rejecting a single locus inheritance model), the best single gene model is dominant inheritance if only BPI is considered[3]. Reported linkages of bipolar affective disorder on chromosomes 11, 18, 21 and X have been difficult to substantiate, and additional studies are required for replication or exclusion of these regions[4–9]. We now present the results of our genome-wide linkage analyses that provide evidence that regions on chromosomes 6, 13 and 15 harbour susceptibility loci for bipolar affective disorder, suggesting that bipolar affective disorder in the Old Order Amish is inherited as a complex trait.

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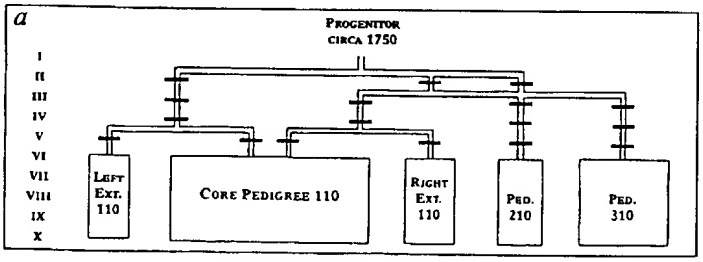
The Amish study has employed rigorous standards for both clinical documentation and independent diagnostic evaluations with various reliability tests (see Methods, and Fig. 1). As the exact mode of inheritance of bipolar affective disorder is unknown, we performed linkage analyses using nonparametric methods (allele sharing, model-independent), as well as lod-score analyses. Sixteen models were tested in the lod score analyses (see Methods).

To evaluate the effect of analysing the data under multiple models, we compiled maximum lod scores for the 551 markers obtained under a single model (Fig. 2, N1)(for dominant inheritance, nuclear families, homogeneity, and affecteds only) and compared them with lod scores obtained under multiple testing (Fig. 2, N2). Mean lod scores were 0.18 versus 0.46, for N1 and N2, respectively. Thus, multiple testing raised lod scores on the average by 0.28 units. Also, no lod score exceeded 2.0 under the fixed model (Fig. 2, N1), whereas three lod scores had values of at least 2.0 under multiple testing (Fig. 2, N2). For the sib-pair analyses (see below), we used two weighting schemes and examined four categories of affection status. Also, the SIBPAL program furnished analyses for the mean number of alleles sharing identity by descent (IBD) in affected sib pairs as well as for regression of the number of alleles sharing IBD on the squared difference of phenotypes in sib pairs, where phenotype = 0 for unaffected and phenotype = 1 for affected. This degree of multiple testing (12 tests) increases the type 1 error. As tests are non-independent it is difficult to judge the inflationary effect of multiple testing but the effect is likely to be moderate as shown by the more formal analysis of multiple lod score tests given above.

We also performed analyses using the affected pedigree member (APM) method and the transmission disequilibrium test (TDT). The APM method furnished a few isolated low empirical *P* values that were not supported by other analyses. Thus, no APM results are given here. To assess a possible role of linkage disequilibrium, *P* values for the TDT were determined.

Eleven percent of the markers (62/551) used in our genome-wide search gave a maximum lod score of 1.0



b Old Order Amish subjects studied in genome scan

	Ped.110 LeftExt	Ped.110 Core	Ped.110 IstRt.Ext	Ped.210 2ndRt.Ext	Ped.310	Total
Diagnoses						
BP I	3	11	4	2	11	31
BP II	0	3	1	1	3	8
MDD:recurrent	1	5	3	2	4	15
MDD:single	1	5	1	1	1	9
Other Dx.	0	9	1	3	5	18
Affected	5	33	10	9	24	81
Unaffected	5	52	21	19	29	126
Grand total	10	85	31	28	53	207

Fig. 1 Ancestral trace for Amish study bipolar pedigrees. *a*, Summary of each pedigree in 'block' form, illustrating that all of the BPI pedigrees trace along pathways leading to a common progenitor, one of some 30 couples that founded the present Lancaster County, Old Order Amish group[36]. This ancestral line encompasses the earliest cases of recurrent, psychiatric illness and the first confirmed cases of bipolar affective disorder. Bipolar I disorder among descendants of other pioneers usually occurred after intermarriage with the BP progenitor line[14]. Note the LEFT extension coupled with the CORE Pedigree 110, which provided the resource first used to report genetic linkage data[4]. After follow-up and addition of a RIGHT extension to Pedigree 110, further genetic analyses were reported in 1989 (ref. 5). Pedigree 210 and partial Pedigree 310 (NIGMS Family 1075)[19] became a second large lateral extension[37]. Our report utilizes all of these earlier subjects plus additional expansions, especially in Pedigree 310. *b*, Summary of the diagnoses for the individuals in this study. One unaffected subject is counted twice — as a sibling/aunt of bipolar affecteds in Pedigree 210, and as a spouse/mother of bipolar children in Pedigree 310.These Old Order Amish kinships continue to provide for lateral and lineal expansion and have evolved into the IX–Xth generations of descendants at risk.

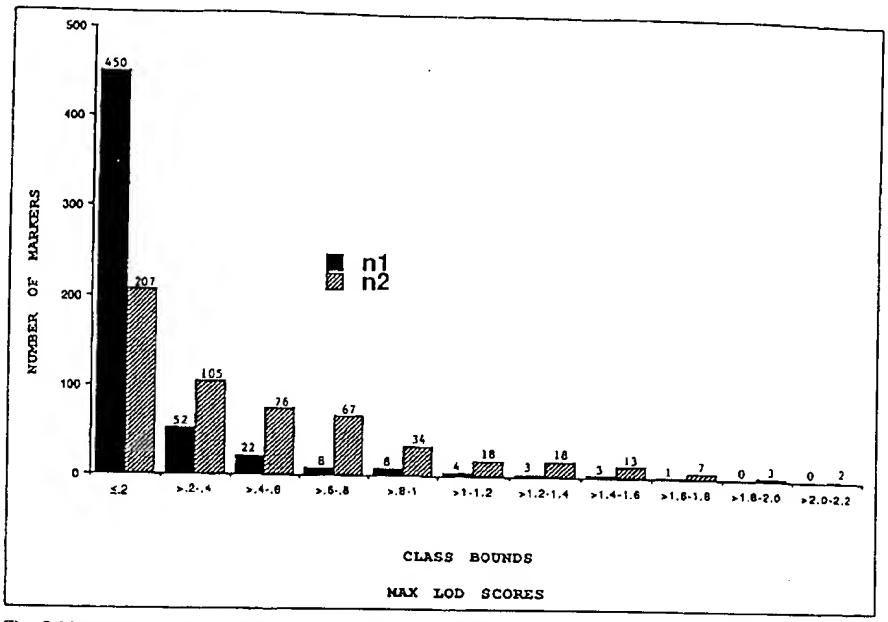


Fig. 2 Maximum lod scores, using BPI as affected, for two scenarios: (N1) nuclear families, homogeneity, dominant inheritance, and affecteds only; and (N2) 16 combinations of analyses for each marker including dominant vs recessive inheritance, 5 pedigrees vs. nuclear families, homogeneity vs. heterogeneity, and affecteds only vs affecteds and unaffecteds. N1 and N2 represent the number of markers furnishing maximum lod scores within given class boundaries for scenarios 1 and 2, respectively.

or higher in at least one of the 16 analysis models. Consequently, only regions which yielded stronger evidence of linkage were considered further, namely those with markers having a test statistic of $P<0.01$ in any one analysis type, or maximum lod score of at least 2.0. Six markers (*D1S48, D6S7, D7S67, D11S146, D13S1* and *D15S45*) met these criteria. Markers at three loci gave P values of 0.001 or smaller under SIBPAL analyses: *D6S7* (chromosome 6pter–p24) with $P = 0.0003$; *D13S1* (chromosome 13q13) with $P = 0.0003$; and *D15S45* (chromosome 15q11–qter) with $P = 0.0003$. Test statistics for these three markers, as well as for markers flanking these regions, are shown in Table 1. Some of the formal P values furnished by the SIBPAL program were re-estimated using computer simulations (3,000 replicates) run under an assumption of no linkage and are indicated by P^{a} (see Methods). Therefore, they are not flawed by analyses that would furnish spuriously small formal P values. In lod-score analysis none of the markers reached lod = 3 criterion. However, in some of the nonparametric analysis methods, P values of less than 0.001 (and even 0.0003, which is asymptotically equivalent to $Z_{max} = 2.5$) were found.

Results are typically stronger for BPI than for more liberal diagnostic categories (Table 1); that is, extending the pool of affected individuals to include additional psychiatric illness (BPII and recurrent MDD) appears to add 'noise' to the analyses. Generally, we obtain equivalent results for lod-score analyses and our ASP analysis, whereas the Haseman-Elston approach (SIBPAL program) typically provided stronger results. The main difference between the two programs, SIBPAL and ASP, is that there is no weighting of multiple sib pairs in a sibship in SIBPAL.

The relationship between pointwise (locus-specific or nominal) and genome-wide significance levels was recently discussed by Lander and Kruglyak[10]. For sib-pair methods, pointwise P values[10] of 0.00074 and 0.000022 correspond to suggestive and significant linkage, respectively, with 'significant' denoting a genome-wide P value of 0.05. For lod score analysis, the respective lod-score thresholds are 1.9 and 3.3.

For *D6S7, D13S1* and *D15S45* (see Table 1) an analysis of allele frequencies was carried out on probands and their mates to test for the existence of linkage disequilibrium[11]. Intraclass correlations for alleles within individuals and between individuals (who are mates) were calculated. In no case was there a significant difference at the 5% level. For these three loci, as well as for the two markers flanking each of them, we tested for the presence of specific haplotypes. Again, no haplotypes were significantly more frequent than expected by chance when tested at the 5% level.

A recently described approach[12] for estimating the proportion of alleles sharing IBD based on all markers of a chromosome, was applied to the chromosomes carrying our best three markers (Table 2). For allele sharing among all possible affected sib pairs, it resulted in suggestive linkage for locus *D6S7*. However, TDT did not provide P values suggestive of linkage when applied to our best three loci and BPI affecteds (Table 2).

According to recently proposed criteria[10], *D6S7, D13S1* and *D15S45* yield locus-specific P values suggestive of linkage. Therefore, it is imperative that the evidence for linkage of bipolar affective disorder to DNA markers on chromosomes 6, 13 and 15 in the Old Order Amish observed here be confirmed and extended in other pedigrees. We are collecting additional members of the Amish pedigrees for analysis of more markers in the critical chromosomal regions.

Table 1 Results of linkage analyses

Locus	Map distance (cM)	Z_{max} dom	Z_{max} rec	SIBPAL *P*	ASP *P*
BPI					
D6S344	0.0	.000	.000	.8729	.2145
D6S7	36.0	2.342	1.456	.0003[a]	.0513
D6S89	17.7	.097	.001	.5567	1.0000
D6S28		.000	.003	.6262	.7800
BPI+II[b]					
D6S344	0.0	.000	.000	.9249	.2341
D6S7	36.0	2.469	1.609	.0000	.0293
D6S89	17.7	.167	.000	.7113	.3230
D6S28		.000	.000	.6272	.7867
BPI+II					
D6S344	0.0	.000	.000	.9249	.2036
D6S7	36.0	1.885	.984	.0003[a]	.1561
D6S89	17.7	.732	.394	.5241	.2743
D6S28		.000	.000	.6874	.6892
BPI+II+MDD					
D6S344	0.0	.000	.000	.8977	.1055
D6S7	36.0	1.606	.795	.0001	.4195
D6S89	17.7	.732	.399	.7126	.1714
D6S28		.000	.000	.6877	.7918
BPI					
D13S221	15.3	.000	.012	1.0000	.9725
D13S171	5.2	.000	.102	.4905	.3736
D13S1	5.1	1.276	1.248	.0003[a]	.0057
D13S218	10.1	.312	.664	.0171	.0641
D13S291		.056	.175	.0865	.3028
BPI+II[b]					
D13S221	15.3	.000	.000	1.0000	.8902
D13S171	5.2	.000	.000	1.0000	.4876
D13S1	5.1	1.402	1.036	.0000	.0056
D13S218	10.1	.494	.423	.0175	.0766
D13S291		.004	.178	.1475	.3998
BPI+II					
D13S221	15.3	.000	.000	1.0000	.7344
D13S171	5.2	.000	.000	1.0000	.4665
D13S1	5.1	1.203	.676	.0090[a]	.0162
D13S218	10.1	.307	.314	.1403	.1484
D13S291		.006	.194	.2384	.4033
BPI+II+MDD					
D13S221	15.3	.000	.000	1.0000	.7672
D13S171	5.2	.000	.204	1.0000	.9429
D13S1	5.1	.000	.043	.0223	.0932
D13S218	10.1	.000	.025	.3007	.3202
D13S291		.000	.008	.2262	.3525
BPI					
D15S45	5.6	1.114	.798	.0003[a]	.0163
D15S117	1.2	.130	.580	.0843	.1660
D15S148	6.1	.338	.610	.0217	.1000
D15S38	0.0	.000	.000	1.0000	.9853
D15S36		.355	.400	.0114	.0862
BPI+II[b]					
D15S45	5.6	1.097	.446	.0018	.0456
D15S117	1.2	.332	.589	.1225	.2346
D15S148	6.1	.752	.613	.0219	.0976
D15S38	0.0	.067	.000	1.0000	.9904
D15S36		.646	.402	.0118	.0844
BPI+II					
D15S45	5.6	.857	.731	.0183	.0399
D15S117	1.2	.089	.726	.0910	.1625
D15S148	6.1	.461	.829	.0123	.0589
D15S38	0.0	.000	.000	1.0000	.8551
D15S36		.368	.292	.0131	.1172
BPI+II+MDD					
D15S45	5.6	1.709	.473	.0032	.0150
D15S117	1.2	.000	.096	.2119	.3546
D15S148	6.1	.148	.182	.0360	.0998
D15S38	0.0	.000	.000	1.0000	.7914
D15S36		.000	.000	.1423	.3168

Z_{max}: maximum lod score in analysis of nuclear families, affected only, penetrance ratio (genetic versus nongenetic cases) of 500:1, with allowance for heterogeneity (exception: for *D6S7*, affecteds and unaffecteds)
Z_{max} dom or Z_{max} rec: under dominant or recessive inheritance
SIBPAL *P*: *P* values furnished by SIBPAL program in t-test for excess allele sharing in affected sib pairs (exception: results for regression analysis given for *D6S7*).
ASP *P*: *P* values in t-test for excess of allele sharing in affected sibs, multiple sib pairs in same sibship weighed by number of meioses
Clinical categories: MDD includes only recurrent major depressive disorder; Number of affecteds in clinical hierachies were: 31 BPI, 35 BPI+BPII[b], 39 BPI+BPII, and 49 BPI+BPII+MDD
[a]Empirical *P* values were estimated by computer simulation
[b]Only those BP II cases that are borderline BP I are included (such as clinical BP I and RDC manic)

Table 2 Test results for TDT and multipoint affected sib-pair analysis for BPI phenotype

	TDT (*P* value)	Sib-pair analysis (lod score)	
		Indep. pairs	All pairs
D6S344	0.063	0.089	2.139
D6S7	0.200	0.920	2.211
D6S89	0.500	0	0.091
D6S28	0.500	0	0.039
D13S221	0.500	0	0.002
D13S171	0.500	0.598	0.772
D13S1	0.500	0.868	1.422
D13S218	0.497	0.595	1.490
D13S291	0.500	0	0.375
D15S49	0.500	0.737	0.517
D15S48	0.500	0.239	0.247
D15S45	0.166	0.877	1.540
D15S117	0.500	0.084	0.661
D15S148	0.119	0.073	0.629

Multipoint affected sib pair analysis was carried out with the MAPMAKER/SIBS program[12], pairing options 2 (independent pairs) and 3 (all possible pairs), with allowance for dominance variance.

Past attempts to replicate linkage findings for bipolar affective disorder have been plagued by diagnostic uncertainties, genetic heterogeneity, phenocopies, genotyping errors and the complexities of performing and interpreting statistical results. Our study, however, represents a 19-year longitudinal study of an isolated population in which there is a relatively narrow spectrum of illness (not one case of schizophrenia occurs in these pedigrees); bipolar disorder is the predominant diagnosis. The rigorous longitudinal assessment of these pedigrees combined with the systematic and blind psychiatric evaluations and diagnoses should also greatly reduce the number of misdiagnoses included in our linkage analyses. Moreover, the restricted gene pool characteristic of this relatively closed population should reduce the number of disease-causing alleles, minimizing the problem of genetic heterogeneity.

Similar to other common and complex diseases like diabetes, hypertension, and perhaps even schizophrenia, our data suggest that genetic factors likely contribute to the pathogenesis of bipolar affective disorder, where in the majority of cases, inheritance is multifactorial rather than simple mendelian transmission. Like the genetic variance observed for quantitative traits, bipolar affective disorder (even in a relative genetic isolate like the Old Order Amish) appears to be a polygenic (complex) trait resulting from the variable effects of multiple genes. The results of our genome wide scan suggest that genes on chromosomes 6, 13 and 15, rather than just different mutant alleles of a single gene, determine the susceptibility to and phenotype of bipolar affective disorder in the Old Order Amish. Additional sets of genes may underlie the susceptibility to develop bipolar affective disorder in other populations. Although we emphasize the provisional nature of these loci, the findings from our genome wide search in the Amish can now be examined in other populations, and may eventually lead to the identification of specific genes responsible for bipolar affective disorder.

Methods

Diagnostic assessment. The genetic-epidemiologic study of bipolar affective disorder among the Old Order Amish in southeastern Pennsylvania has been described[13,14]. Case ascertainment for mental illness among the Amish began with a community-wide network of informants and institutional rosters reviewed with informed consent[15]. Over 400 patient cases

have been ascertained. A psychiatric review board composed (since 1976) of James N. Sussex, Abram M. Hostetter, John J. Schwab, David R. Offord and Jean Endicott used both psychiatric interviews[16] and abstracted medical records to perform diagnostic assessments based on strict research diagnostic criteria (RDC)[17]. Assessments by this review board were made blind to pedigree membership, diagnostic opinions and treatment information in the medical records, and genetic marker status. As the Board's diagnostic procedures yielded confirmed cases of BPI affective disorder, the immediate families of these patients were evaluated for psychopathology. Pedigree 110 was selected (in 1981) for initial genetic linkage study because of relationships between nuclear families, based on BPI probands, and illness spanning several generations[14]. When one examines the relative risk for individuals used in our linkage study, there is a very high prevalence of affective disorder, with age-corrected morbid risk rates for BPI, BPII, and MDD (major depressive disorder) of 17%, 4% and 6%, respectively. This gives an overall rate of 27% for major affective disorder in our pedigrees. The present sample, which includes extensions to the original family (Fig. 1*a*) totals 207 members, with 31 diagnosed BPI, 50 with other psychiatric diagnoses, and 126 unaffected individuals (Fig. 1*b*). Over 125 medical records were abstracted and Board reviewed to document the 31 BPI cases. The average age of onset for BPI disorder was 22 years. Reliability of the bipolar diagnoses was checked when 16 of the 31 cases (52%) were evaluated twice, with an average five year interval between the blind assessments using different clinical documentation and resulting in 100% concordance. The high reliability obtained lessens the likelihood of misdiagnoses or a false positive BPI in our linkage analyses[18].

Apart from RDC diagnoses, the project psychiatric panel also recorded clinical opinions in a consensus 'clinical diagnosis'. There was 100% concordance between these two types of diagnostic conclusions (5 board members) for the 31 BPI cases and 13 of the 15 cases of recurrent major depressive disorder (Fig. 1*b*). Of particular interest are the diagnostic results for the 8 cases of BPII. Four were designated BPII by both RDC and clinical opinion. The other four were labelled BPI according to clinical opinion, and two were classified as 'probable BPI' by the strict RDC. This is important because it shows that true BPII disorder occurs rarely in these pedigrees; BPII appears more as a 'BPI' waiting to happen.

Patient samples. Only after the initial diagnostic assessments of probands and their relatives were the blood samples obtained from the subjects using informed consent. Codes were used to maintain confidentiality. The phlebotomist was kept blind to pedigree relationships and diagnostic status. Given a variable age of onset for the affective disorders and the impact of new diagnoses on data analyses, the Amish Study policy is to conduct annual follow-up. This permits an update of any changes in the course of illness for affected individuals and allows us to see if previously well individuals have onset with illness. It also facilities ascertainment of new cases of major affective disorder in close branches of the family so plans can be made to include them in pedigree expansion. Blood samples were collected with informed consent and lymphoblastoid cell lines were established at the Coriell Institute of Medical Research and/or the National Institute of Mental Health. The NIGMS Human Genetic Mutant Cell Repository catalog contains updated pedigree and diagnostic information[19].

Genotyping. DNA was extracted from peripheral blood samples and/or immortalized lymphoblastoid cell lines[20]. Mapping panels were constructed to determine the best order of markers typed on the bipolar pedigrees using genotypic data from the CEPH version 7 database, using the MultiMap linkage analysis program[21]. The RFLP and microsatellite markers used resulted in a linkage map with an average spacing of between 5 and 10 cM[22,23]. Microsatellite markers were genotyped individually as described[9,24], and by an adapted multiplex procedure[25]. In the multiplex genotyping procedures, a total of 25 markers were analysed in each lane of the genotyping gels. To accomplish this, five markers were coamplified in each PCR tube, and five sets of five markers were pooled and precipitated prior to gel loading. Sets of five markers were amplified in 20 µl reactions that included: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 1.5 mM $MgCl_2$, 0.2 mM dNTPS, 0.5 U *Taq* polymerase, 1 µM of each primer (10 primers in total), and 50 ng genomic DNA. Samples were denatured at 94 °C for 1 min, followed by 30–35 cycles at 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s. After the final cycle, the reactions were incubated at 72 °C for 3 min. Following amplification, five sets of PCR amplifications were pooled and isopropanol precipitated in 96 well microtiter plates. The pooled PCR products were resuspended in 10 µl loading dye containing formamide, denatured at 95 °C for 5 min and loaded onto 6.0% denaturing polyacrylamide gels. After electrophoresis, the gel fractionated microsatellite markers were transferred to nylon membranes by capillary transfer, and visualized by hybridizing membranes with marker specific, chemiluminescent probes. To make the probes, one of the oligonucleotides used to amplify each marker was labelled using the ECL detection kit (Amersham). Multiple probes corresponding to markers of different sizes were hybridized to the filters simultaneously. Chemiluminscent signals were detected by autoradiography. Allele sizes for the microsatellite markers were determined relative to a *pUC18* sequence or Sequamark marker ladder (Research Genetics). To maintain allelic designations for the purpose of allele frequency calculations, DNA samples from replicate individuals were included within and between gels. Films were scored either manually or using semi-automated allele-calling software (BioImage), and were independently analysed by two individuals blind to disease status. Data from all markers were transferred into the same file system for linkage analyses.

Linkage analyses. Statistical analyses for linkage were performed using both nonparametric (SAGE SIBPAL program for Haseman-Elston sib pair tests; affected sibpair analysis (ASP) with weighting of multiple affected in the same subship by number of meioses; affected pedigree member (APM); and the transmission disequilibrium test (TDT)) as well as lod score analyses. Sixteen models were tested in the lod score analyses, and included: dominant versus recessive inheritance; large pedigrees versus data broken into nuclear families; homogeneity versus allowing for heterogeneity; and analysis for affecteds only versus using affecteds and unaffecteds. For nonparametric analyses, based on the asymptotic (theoretical) distribution of the test statistic, the SIBPAL program[26] furnished formal *P* values in the test for an excess proportion of alleles shared IBD and in the Haseman-Elston regression test[27]. The 'true' *P* value (the empirical significance level) is defined as the probability that the observed result, or more extreme one, is obtained by chance alone. To estimate empirical significance levels associated with these results rather than relying on the formal *P* value, we carried out computer simulations (3,000 replicates) for each marker with a formal *P* value of 0.01 or less. These simulations were extremely time consuming as a complete analysis had to be carried out for each replicate. Therefore, empirical *P* values were obtained only for those analyses on the BPI and BPI + II clinical diagnostic categories where markers had a formal *P* value of 0.001 or less. Resulting empirical *P* values (identified by P^a in Table 1) typically were about ten times higher than the formal '*P* values' issued by the program. In addition, sib-pair analyses were performed using a computer program in which multiple pairs per sibship are weighted by the number of meioses, and that also carried out the TDT analyses[28–31]. Multiple alleles were taken into account as described in an extension to the TDT[32]. The TDT was applied to allow for a possible involvement of linkage disequilibrium. The total number of affected sib pairs in our data set was 12 for BPI, 13 for BPI +

BPII[b] (borderline BPI), 19 for BPI + BPII, and 30 for BPI + BPII + MDD. While these numbers are small, they do not represent random sib pairs but rather come from a unique closed population and are, presumably, all at least distantly related to each other.

Allele sharing based on all markers on a chromosome was applied as implemented in the MAPMAKER/SIBS program, resulting in lod scores along points on the chromosome. Two program options for forming affected sib pairs were used, 'independent pairs' (first sib versus each of the other sibs) and 'all pairs'. However, because sib pairs are unweighted, the latter option may furnish lod scores that are too high in small to moderate sample sizes.

For parametric analyses, two-point lod scores were calculated with the LINKAGE programs[33]. Each marker was analysed under 16 models (dominant versus recessive inheritance, large pedigrees versus data broken into nuclear families, homogeneity versus allowing for heterogeneity by the HOMOG program[34], and for affecteds only versus affected and unaffected individuals considered). A penetrance ratio (genetic versus non-genetic cases) of 500:1 and disease allele frequencies adjusted to reflect a population prevalence of 1% were used. Individuals without psychiatric illness under any diagnostic scheme were considered unaffected, whereas those not categorized as affected under one scheme but affected under one of the other diagnostic categories were taken to be unknown.

Linkage disequilibrium influences certain types of identity-by-state affected sib-pair strategies[35]. The basic reason for this is that allele sharing among relatives in isolated populations may be exaggerated due to the fact that large regions of founder chromosomes have not been broken up by recombination. For this reason, we employed the TDT to test for linkage, and applied IBD methods, which are unlikely to be significantly influenced by disequilibrium.

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Melanocortin-4 receptor gene variant i103 is negatively associated with obesity.

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Several rare mutations in the melanocortin-4 receptor gene (MC4R) predispose to
obesity. For the most common missense variant V103I (rs2229616), however, the
previously reported similar carrier frequencies in obese and nonobese individuals are in line with in vitro studies, which have not shown a functional
implication of this variant. In the present study, we initially performed a
transmission/disequilibrium test on 520 trios with obesity, and we observed a
lower transmission rate of the I103 allele (P=.017), which was an unexpected
finding. Therefore, we initiated two large case-control studies (N=2,334 and
N=661) and combined the data with those from 12 published studies, for a total
of 7,713 individuals. The resulting meta-analysis provides evidence for a
negative association of the I103 allele with obesity (odds ratio 0.69; 95%
confidence interval 0.50-0.96; P=.03), mainly comprising samples of European
origin. Additional screening of four other ethnic groups showed comparable I103
carrier frequencies well below 10%. Genomic sequencing of the MC4R gene revealed
three polymorphisms in the noncoding region that displayed strong linkage
disequilibrium with V103I. In our functional in vitro assays, the variant was
indistinguishable from the wild-type allele, as was the result in previous
studies. This report on an SNP/haplotype that is negatively associated with
obesity expands the successful application of meta-analysis of modest effects in
common diseases to a variant with a carrier frequency well below 10%. The
respective protective effect against obesity implies that variation in the MC4R
gene entails both loss and gain of function.

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Apolipoprotein-E ε4 Allele Frequency and Conferred Risk for Cuban Americans With Alzheimer's Disease

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The authors compared Apo-E ε4 frequencies for Cuban American and white non-Hispanic persons with and without Alzheimer's disease (AD). The Apo-E ε4 allele conferred as large a risk for AD in Cuban Americans as for white non-Hispanics, a finding that differs from those for Hispanic subjects as a whole. (Am J Geriatr Psychiatry 2000; 8:254-256)

An association between Alzheimer's disease (AD) and the apolipoprotein-E (Apo-E) ε4 allele has been established worldwide.[1] The strength of the association, usually measured by comparing Apo-E ε4 allele frequencies for patients with AD with those for age-matched control subjects without dementia, has been reported to vary across ethnicities and geographical areas.[2] The ethnic variations in Apo-E ε4-conferred risk for AD are potentially important because they may correspond to ethnic differences in pathogenetic mechanisms[1] or in clinical manifestations of the disease.[3]

Cross-ethnic comparisons of Apo-E ε4 allele frequency in patients with AD in the United States have typically utilized a three-way ethnic partition, distinguishing white non-Hispanics, Hispanics, and African Americans as distinct subgroups. Using this scheme, assessments of Apo-E ε4-conferred risk for AD by Tang et al.[4] and Farrer et al.[2] revealed a lower risk for Hispanic than for white non-Hispanic individuals. More recently, Tang et al.,[1] utilizing data from a prospective longitudinal study, made the stronger argument that the Apo-E ε4 allele conferred no risk of AD at all for Hispanic individuals. The low or absent Apo-E ε4-associated risk for AD reported for Hispanic individuals may not, however, be generalizable to all Hispanics but may be different for different Hispanic subgroups.

In the present study, we took advantage of the availability of a homogeneous Hispanic subgroup comprising Cuban American individuals living in South Florida. We evaluated Apo-E genotypes for Cuban American and white non-Hispanic patients with AD and age-matched control subjects without dementia and compared the risk for AD conferred by the Apo-E ε4 allele for both groups.

METHODS

A total of 145 patients with AD (80 Cuban American and 65 white non-Hispanic) and 49 control subjects (21 Cuban American and 28 white non-Hispanic) served as the study group. Patients with AD were chosen consec-

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utively from patients diagnosed with probable AD according to NINCDS criteria[5] after a comprehensive clinical and laboratory assessment conducted by the State of Florida-funded University of Miami Memory Disorders Clinic. Control subjects were either spouses (83%) or friends (17%) of patients. Patient ethnicity was determined by self-report (or caregiver report) of ethnicity ("Hispanic" or "white non-Hispanic") and country of birth. All subjects in the Cuban American group classified themselves as Hispanic and gave their country of birth as Cuba. Age at disease onset for AD patients was estimated via structured interview of the patient's caregiver by two independent examiners (interrater Pearson $r = 0.85$). The average of the two estimates was used for analyses.

Apo-E genotypes were determined by PCR amplification according to the method previously described by Bennett et al.[6] Allele frequencies were determined by counting alleles and calculating proportions relative to the total number of alleles in the sample. Chi-square was used to compare Apo-E ε4 allele frequencies for patients with AD with those for control subjects for both the Cuban American and white non-Hispanic groups. To determine whether the increase in Apo-E ε4 frequency for patients with AD relative to controls was different between ethnicities, a method for comparing odds ratios described by Cohen[7] was used. First, phi-coefficients (ϕ; i.e., the fourfold point correlation coefficient) were calculated for the contingency tables corresponding to each ethnicity; then a Fisher z transformation was applied; and finally, the statistical significance of the difference between the transformed correlation coefficients was evaluated with N set as the harmonic mean of the ns for the two groups.

RESULTS

There were no significant differences in age or sex between Cuban American and white non-Hispanic subjects for either patients or control subjects. Also, there was no significant difference between Cuban American and white non-Hispanic patients with AD for estimated age at disease onset. Patients and control subjects were matched for age, but patients with AD were more likely than control subjects to be female ($P = 0.005$).

The Apo-E genotypes and allele frequencies for Cuban American and white non-Hispanic patients with AD and control subjects are shown in Table 1. Apo-E ε4 allele frequencies were greater for patients with AD than for control subjects, both for white non-Hispanic ($P = 0.005$) and for Cuban American subjects ($P = 0.01$). The relative risk of AD conferred by the Apo-E ε4 allele was somewhat greater for Cuban Americans (OR = 4.33; range: 1.27–14.79) than for white non-Hispanics (OR = 3.40; range: 1.48–7.79), but the difference was not statistically significant ($P > 0.1$).

DISCUSSION

For both Cuban American and white non-Hispanic groups, the Apo-E ε4 allele frequency was greater for

TABLE 1. Apo-E genotype and allele frequencies, *n* (percent)

	Cuban American		White Non-Hispanic	
	AD ($n = 80$)	Control Subjects ($n = 21$)	AD ($n = 65$)	Control Subjects ($n = 28$)
Genotype				
4/4	6 (7.5)	0 (0)	8 (12.3)	1 (3.6)
4/3	28 (35.0)	2 (9.5)	28 (43.1)	5 (17.9)
4/2	0 (0)	1 (4.8)	3 (4.6)	1 (3.6)
3/3	40 (50.0)	17 (81.0)	19 (29.2)	18 (64.3)
3/2	7 (7.5)	1 (4.7)	7 (10.8)	3 (10.6)
2/2	0 (0)	0 (0)	0 (0)	0 (0)
Allele frequency				
2	0.037	0.48	0.077	0.071
3	0.713	0.881	0.562	0.786
4	0.250	0.071	0.361	0.143
Odds ratio		4.33		3.40
95% Confidence limits		1.27–14.79		1.48–7.79

Note: AD = Alzheimer's disease.

patients with AD than for control subjects without dementia, indicating that the Apo-E ε4 allele conferred a risk for AD for both groups. Comparison of the phi-coefficients for the corresponding contingency tables indicated that the risk was not significantly different between the two groups. The substantial risk of AD conferred by the Apo-E ε4 allele for Cuban Americans in this study contrasts with the previously reported low or nonexistent risk of AD associated with the Apo-E ε4 allele for Hispanics in North Manhattan[1,4] or of unspecified composition.[2] It is possible that differences in the ethnic makeup of the Hispanic groups in those studies and the present one are at least partly responsible for the differences in findings. Schur et al.[8] have noted that it is important to distinguish among such Hispanic subgroups as Cuban American, Mexican American, and Hispanics of Puerto Rican or Dominican origin when conducting clinical studies comparing Hispanic and non-Hispanic groups. The findings in the present study suggest that such a distinction may be necessary when assessing the implications of Apo-E ε4 allele frequency and conferred risk for AD.

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Identification of a protective allele against Alzheimer disease in the APOE gene promoter

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Alzheimer disease (AD) risk is significantly influenced by the APOE2 and APOE4 alleles. In turn, the −491AT and TH1/E47cs polymorphisms alter APOE gene expression levels. To determine whether these two alleles exert any significant effect on AD development we have analysed the genotypes of the APOE promoter −491AT and Th1/E47cs polymorphisms in 163 AD patients and 155 controls divided into three age at onset/age dependent subgroups. Our study has detected a Th1/E47cs-T allele accumulation in healthy individuals over 75 years of age, which suggests it plays a protective role against AD. The Th1/E47cs-T allele may provide greater protection against AD than APOE2, although this awaits proof of Th1/E47cs-T allele overrepresentation in healthy individuals of other populations. *NeuroReport* 13:1403–1405 © 2002 Lippincott Williams & Wilkins.

Key words: Alzheimer disease; Apolipoprotein E; APOE promoter region polymorphisms; Protective allele

INTRODUCTION

The apolipoprotein E (APOE) gene is known to be a major risk factor for AD in many populations [1,2]: the APOE4 allele increases the risk of AD in a dose-dependent manner [3,4] and the APOE4/4 genotype may precipitate the onset of the disease [4]. On the other hand, since the APOE2 allele shows a high prevalence among centenarians [5,6], some authors have concluded that it protects against AD [7,8]. Statistically, whereas AD will develop in 50% of APOE4/4 homozygotes before they reach 70 years of age, the median age at onset of AD among APOE2/3 heterozygotes is over 90 years [9]. Moreover, in contrast to the fast rate of cognitive decline shown by APOE4/4 patients, the APOE2 allele is associated with slow AD progression [10].

A flanking markers study has suggested that polymorphisms in the APOE locus itself may also confer risk for developing AD [11]. This hypothesis has gained support from the observation of an allelic distortion in relative APOE mRNA levels in the brain of AD patients as compared with controls [12]. Several polymorphisms in the APOE promoter region have also been identified [13–15]. Two of these polymorphisms (−491AT and Th1/E47cs) influence APOE gene expression levels, which are diminished by the −491-T allele and increased by the Th1/E47cs-G allele [14].

Different studies have reported a deleterious effect of the Th1/E47-T allele [15] and a protective effect of the −491-T allele, the −491-AA genotype being responsible for increased risk in AD patients [13]. An additional study has confirmed the deleterious effect of the Th1/E47-T allele and the protective effect of the −491-T allele but only in APOE3/4 genotype carriers [16]. If these alleles alter APOE gene transcription levels, the risk conferred by the APOEε4 allele or the protective effect exerted by the APOEε2 allele should vary accordingly [15].

Several authors have proposed the existence of protective genes against AD. In this regard, AD prevalence has been found to be decreased in a cohort with a high degree of Cherokee Indian ancestry studied in Oklahoma [17]. The low frequency of AD in this population was not changed by the APOEε4 allele. Similar data have been reported when the Cree Indian tribe was compared with an urban white population [18].

Since AD risk is significantly influenced by the APOE and APOE4 alleles and, in turn, APOE gene expression is altered by the polymorphism −491AT and Th1/E47cs in the APOE gene promoter region, we have studied these two polymorphisms in three age at onset/age dependent subgroups of AD patients with the aim of detecting differences in allele representation between patients and controls.

MATERIALS AND METHODS

The study included 163 AD patients (age range 48–85 years; mean age 70.8 years; male:female ratio, 1: 1.6) with clinical diagnosis of probable AD according to the DSM-IV and NINCDS-ADRDA criteria [19]. Also investigated were 155 control subjects (age range 45–84 years; mean age 68.7 years;

male:female ratio, 1:1.5) with no neurodegenerative disorder. Patients and controls were divided into subgroups according to their age at onset/age as follows: <65 years (46 patients, 46 controls), between 65 and 74 years (69 patients, 65 controls), and ≥ years (48 patients, 44 controls).

DNA was extracted from peripheral blood cells according to standard procedures. The common APOE gene polymorphism was studied as described previously [20]. A nested PCR was used for promoter polymorphism detection. First, a fragment of 418 bp was amplified with primers APOE491A: CACCATgTTggCCAggCTggTCTCAA and TH1/E47L: ggAggAAggAggTggggCATAgAgg. PCR reaction mix contained 5 mM $MgCl_2$, and annealing and extension were carried out in a single step of 1 min at 70°C. The obtained fragment served as template for amplification of two fragments containing the −491AT and Th1/E47 polymorphisms as described [14].

Allele frequencies were calculated by the allele counting method. The χ^2 test of association was employed for the assessment of allele frequency differences between controls and patients, whereas calculation of odds ratios by logistic regression analysis was used to determine the effects of age, gender, and the −491 AT and Th1/E47cs polymorphisms on healthy controls. A and T allele frequency values were used for −491AT polymorphism analysis, whereas the Th1/E47cs polymorphism was evaluated by means of T and G allele frequency values. Correction for the presence of both the −491-Tand Th1/E47cs-G alleles in APOE3/3 and APOE4/4 homozygous individuals was applied.

RESULTS

When the AA, AA/AT, TT, or TT/AT genotype frequencies of the −491 AT polymorphism and the TT, TT/TG, GG, or GG/TG genotype frequencies of the Th1/E47cs polymorphism were analysed, the results obtained did not reveal any significant differences in genotype distribution between patients and controls for either the −491 polymorphism or Th1/E47 polymorphism. Taking into account that the −491-T allele diminishes APOE gene transcription and the Th1/E47-G allele enhances it, corrections were applied for the presence of both the −491-T and Th1/E47-G alleles in APOE 313 and APOE 4/4 homozygous carriers in our data analysis.

The −491 AT polymorphism exhibited T-allele frequencies lower than 0.1 in patients and between 0.09 and 0.13 in controls, with no significant differences among the various age at onset-dependent/age subgroups (Table 1).

Th1/E47 polymorphism allele frequencies did not show significant differences between patients and controls in subgroups including individuals up to 74 years of age. On the contrary, the control subgroup formed by subjects of 75 years or older exhibited a very high Th1/E47-T allele frequency when compared with patients and the other age-dependent control subgroups. Testing of the association between the Th1/E47-T allele and lack of AD provided a high odds ratio for the subgroup consisting of individuals aged ≥75 years (Table 2).

As expected, APOE allele frequencies did not present significant differences between cases and controls. Frequency-values were 0.02, 0.03 and 0.03 for the three AD subgroups and 0.06, 0.05, and 0.06 for the control subgroups.

Table 1. −491AT polymorphism allele frequencies among AD cases and controls

Group	Number	Alleles			
	AD/C	A (AD/C)	T (AD/C)	p[a]	OR[b]
<65	46/46	0.94/0.91	0.06/0.09	0.294	n.s.
65–74	69/65	0.95/0.90	0.05/0.10	0.096	n.s.
>75	48/44	0.93/0.87	0.07/0.13	0.124	n.s.

[a]Value for χ^2 test of differences in −491 AT polymorphism allele frequencies between cases and controls.
[b]Odds ratios (OR) estimates for the effect of the −491-A allele on risk for AD.

Table 2. Th1/E47cs polymorphism allele frequencies among AD cases and controls

Group	Number	Alleles			
	AD/C	A (AD/C)	T (AD/C)	p[a]	OR[b]
<65	46/46	0.59/0.55	0.41/0.45	0.421	n.s.
65–74	69/65	0.52/0.44	0.48/0.56	0.107	n.s.
>75	48/44	0.48/0.83	0.52/0.17	<0.001	4.8 (2.3–9.4)

[a]Value for χ^2 test of differences in Th1/E47cs polymorphism allele frequencies between cases and controls.
[b]Odds ratios (OR) estimates for the effect of the Th1/E47-G allele on risk for AD.

These values are in agreement with known APOE allele frequencies in AD patients and the normal population.

DISCUSSION

The results yielded by the present study allow us to propose the Th1/E47-T allele as a protective factor against AD in individuals ≥75 years of age. Our data are in disagreement with those obtained by Lambert *et al.*, who have reported a deleterious effect for the same allele [15]. These discrepancies are probably due to differences in data analysis. Specifically, in the study by Lambert *et al.* which included 293 AD patients (age at onset 71.02±8.9 years; range 49–94) and 310 controls (age 73.5±10.9 years; range 59–105), division into age at onset/age dependent subgroups was not introduced and correction for the presence of both the −491-Tand Th1/E47-G alleles in APOE3/3 and APOE4/4 homozygous individuals was not applied (the existence of the −491 AT polymorphism was then unknown) [15].

Formation of amyloid plaques and neurofibrillary tangles, the neuropathological hallmarks of AD, precedes by many years the appearance of the first AD symptoms. In fact, these neuropathological changes have become irreversible by the time the disease is diagnosed [21,22]. Among the many molecules taking part in the formation of amyloid plaques, apoE outstands for being essential for β-amyloid deposition and insoluble fibril formation, as shown by data obtained in an APOE knockout mouse model studies [23,24]. Since APOE gene expression is inhibited by the Th1/E47-T allele and enhanced by the Th1/E47-G allele [14], apoE levels are lower in the brains of the rare Th1/E47-T allele carriers than in those of the commoner Th1/E47-G allele carriers. Decreased apoE levels would lead to diminished interaction between apoE and β-amyloid in T allele carriers, whose resilience to fibril formation would

result in the slowing-down of amyloid plaque generation and, ultimately, increased resistance to AD development.

Our present results need confirmation by studies of healthy subjects >90 years of age. If similar data are obtained in other populations, the Th1/E47cs-T allele would emerge as an important protective factor against AD. In fact, the protective role of the Th1/E47cs-T allele would be even greater than that of the APOE2 allele, since the former seems to exert its beneficial influence against AD development already at 75 years of age whereas the latter is unable to provide protection until individuals have surpassed 90 years of age.

CONCLUSIONS

The Th1/E47cs-T allele of the APOE promoter region seems to be a protective factor against AD development in the Spanish population. This effect appears to be the result of lower APOE gene expression in Th1/E47cs-T allele carriers in comparison with the commoner Th1/E47cs-G allele carriers. Lower apoE accumulation rates in the brain would slow down amyloid plaque formation and, consequently, provide protection against AD development.

The Th1/E47cs-T allele may well be a protective factor against AD of greater importance than APOE2, although this awaits proof of Th1/E47cs-T allele over-representation in healthy individuals of other populations.

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Apolipoprotein E and Alzheimer's Disease: A Review of Recent Studies

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HIGGINS, G. A., C. H. LARGE, H. T. RUPNIAK AND J. C. BARNES. *Apolipoprotein E and Alzheimer's disease: A review of recent studies.* PHARMACOL BIOCHEM BEHAV 56(4) 675–685, 1997.—There are three isoforms of the 33-kDa protein apolipoprotein E (apoE), termed apoE2, apoE3, and apoE4, each encoded by distinct genes APOE2, APOE3, and APOE4, respectively. In 1993, the APOE genotype was identified as a risk factor for Alzheimer's disease (AD) and was subsequently acknowledged to account for approximately 60% of all cases. The influence of the APOE genotype in AD is clearly isoform dependent, APOE4 imparting susceptibility and APOE2 protection. Thus, patients homozygous for the E4 allele show a very strong likelihood of developing the disease by age 75, whereas patients carrying at least one E2 allele are unlikely to develop symptoms of AD by this age. A major issue in AD research is therefore to understand the functional differences between the ApoE isoforms, with the ultimate aim of designing the next generation of drugs to treat this disease. The purpose of the present article is to summarise some of this work. This review encompasses the rapidly developing molecular, cellular, and behavioural research into ApoE, and attempts to highlight those findings we consider to be of particular significance. © 1997 Elsevier Science Inc.

Alzheimer's disease ApoE Cell biology Transgenics Repair CNS Cognition

IN 1993, Allen Roses and colleagues identified APOE4, the allele encoding apolipoprotein E4 (apoE4), as a susceptibility gene for late-onset Alzheimer's disease (AD) (14,86). Thus, subjects homozygous for APOE4 (APOE4/4) are at much greater risk of developing AD at a particular age than subjects heterozygous for APOE4 (APOE3/4 or APOE2/4), who in turn are at greater risk than those carrying APOE3 or APOE2 alleles. It also appears that subjects carrying one or two APOE2 alleles are "protected" from the disease, such that the likelihood of developing AD increases much later in life than their APOE3 or APOE4 counterparts (13) (see Fig. 1). These findings have now been confirmed by many other workers [see (7,36,60,81,90,98)] and have triggered a wealth of interest in investigating the possible role of apoE in the brain, and in particular, its role in the development of AD.

Alzheimer's disease is characterised phenotypically by a progressive cognitive decline, in particular a loss of memory, learning, and attention (31,57,68). Pathologically, AD is confirmed postmortem by the presence of neuritic amyloid plaques and neurofibrilliary tangles (11,84,92). Histological and brain imaging studies reveal significant brain shrinkage, particularly of temporal lobe structures (43,69,91), and this corresponds with morphological changes also reported in this region, including decreases in dendritic arborisation (17) and loss of neurones and synapses (27,54,109). Regions that show the most significant pathology include the temporal lobes and the frontal, parietal, and posterior cingulate cortices (9,15), areas associated with cognitive function. It now appears that loss of synapses and neurofibrillary tangles correlate best with the progressive cognitive decline (3,9,15,85,109), indicating that neuronal dysfunction is likely to be one of the earliest pathologic mediators of the symptomatology. Thus, research on apoE has focussed largely on investigating its effects on neuronal function and dysfunction, using both in vitro systems and in vivo models. In this review, we will discuss the knowledge to date and consider the evidence suggesting apoE as both a physiological mediator of neuronal function and a key mediator of pathologic development of AD.

CNS DISTRIBUTION

Many of the early investigations into the role of apoE in the brain and AD were considered in the context of the well-established functions of apoE within the periphery [see (47,114)], and it does appear that there are some consistent parallels, although interesting differences are emerging. In particular, the cellular distribution of apoE within the brain is opening new ideas as to the possible function of this ubiquitous protein. Thus, plasma apoE originates from many peripheral organs, particularly the liver, and is known classically to be

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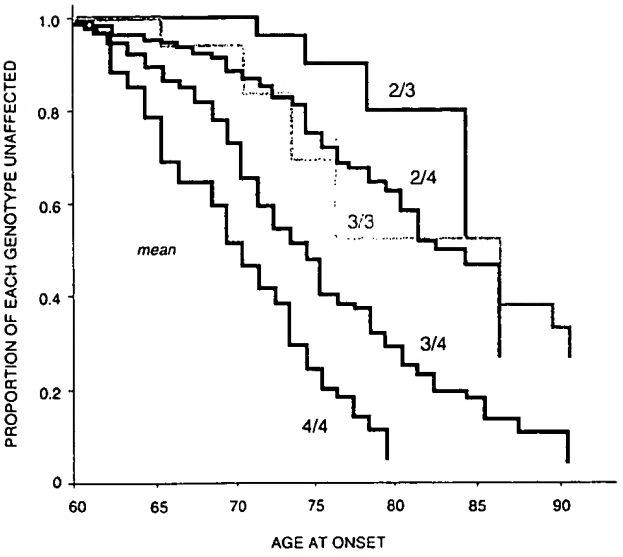

FIG. 1. Graph to illustrate the influence of the APOE genotype on age of onset for Alzheimer's disease. Onset curves were determined by Kaplan–Mayer product limit distributions. An indication of the influence of the APOE genotype on AD onset may be seen by comparing incidence rates at age 75; thus, approximately 80% of subjects homozygous for APOE4 (4/4) were diagnosed with AD, compared with about 40% of subjects homozygous for APOE3 (3/3). The incidence of AD in patients carrying an APOE2 allele is extremely rare, particularly in 2/2 and 2/3 subjects. [Figure reproduced from reference (82) with permission of the author.]

involved in the transport of lipoproteins containing cholesterol and lipids to various cells. This function is mediated by the binding of apoE to two different receptors: the low density lipoprotein (LDL) receptor and the LDL receptor-related protein/α2-macroglobulin receptor (LRP). The brain is the second most important site for apoE biosynthesis, and for many years apoE has been considered as an astrocytic protein (47,95), with a key role in the transport of cholesterol. Recent studies now reveal that, in addition to its expression in astrocytes, apoE can be expressed in neurones, although there appear to be considerable species differences. Thus, in rats and mice, apoE-like immunoreactivity does not normally appear to be expressed in neurones, but rather in microglia and astrocytes. However, in primates (88) and humans (28,29), apoE-like immunoreactivity is found in both astrocytes and neurones. In humans, apoE localises to neurones both in normal brain and in AD brain. In AD brain, apoE can be detected in neurones both with and without neurofibrillary tangles (29), and it has recently been shown that the intensity of apoE-like immunoreactivity appears to occur in areas of the brain showing the most severe pathology (Einstein et al., unpubl. data). At the subcellular level, apoE has been demonstrated in several compartments, including the cytoplasm (28,29).

The difference between species in the cellular localisation of apoE is also confirmed by studies in transgenic mice, which express the different human apoE isoforms in the absence of the native apoE [(117); see *Transgenic Research* section below]. In mice expressing either apoE2, apoE3, or apoE4, apoE can be detected in both neurones and glia, a pattern of immunoreactivity similar to that for the primate. It is still unclear whether apoE is made in neurones rather than being simply transported into neurones from another synthetic site. Indeed, brain neurones do express LRP receptors (10,79,110,115) as well as another member of the LDL receptor family, the very low density lipoprotein receptor (VLDLR) (41), and it is suggested that these receptors may mediate the uptake of apoE into neurones. The role of the LDL receptor in mediating neuronal uptake of apoE is unclear, as the neuronal expression of the LDL receptor remains controversial (79,108). To date, there has been no evidence from in situ hybridisation techniques for the localisation of mRNA for apoE in neurones (16).

CELLULAR AND MOLECULAR INTERACTIONS

Much of the difficulty in assessing the role of apoE in AD stems from the diversity of interactions that the protein demonstrates within the central nervous system (CNS). In the periphery, the story is fairly clear: apoE mediates the transport of lipid between and within cells [see (47)]. However, even this role has yet to be definitively proven in the CNS. There are practicalreasons why it has been difficult to determine the function of apoE in the CNS: the protein is only weakly expressed in rodent brain under normal physiological conditions, so its distribution and site of synthesis, as described in the preceding section, remain controversial. Secondly, in vitro studies are hampered by the inherent stickiness of the protein, which may favour interactions that do not occur under physiological conditions in vivo.

Interaction with Microtubule Associated Proteins

ApoE3 is able to form sodium dodecyl sulfate-resistant complexes with two microtubule associated proteins, tau and MAP2c, in vitro; apoE4 is unable to form these stable complexes (106). MAP2c and tau promote the binding and assembly of microtubule structures, and thus are intrinsic to the stabilisation of the neuronal cytoskeleton (23). The formation of paired helical filaments and neurofibrillary tangles is a hallmark of AD; therefore, it has been proposed that the increased risk of AD in people carrying the APOE4 allele may arise from an inability to maintain microtubule integrity (104).

A feature of the cytoskeleton in the AD brain is the presence of hyperphosphorylated tau. It was originally proposed that hyperphosphorylation would occur because tau was not protected by binding of apoE, and that less protection would be afforded by apoE4 than by apoE3, as discussed above. However, formation of the apoE–tau complex is also dependent upon the phosphorylation state of tau, because phosphorylation of a site within the microtubule binding domain of tau has been shown to prevent binding of apoE (37). Thus, one might envisage that loss of apoE binding and phosphorylation of tau would conspire to cause a rapid breakdown of the neuronal cytoskeleton.

In addition to the formation of a stable complex between apoE and tau, a reversible complex, which may be formed equally by apoE3 and apoE4, has been shown (103). If the formation of this complex is more important in vivo, then a rationale for the increased risk of AD conveyed by the APOE4 gene is diminished; however, the difference between apoE4 and apoE3 could arise from a difference in subcellular localisation rather than any isoform-specific interaction.

Both the stable and reversible interactions of apoE with the cytoskeleton have been shown in vitro, so it will be necessary to demonstrate that either one or both occur under physiological conditions. In rodents, there is an apparent disparity between

the level of apoE expression in neurones compared with that for tau or MAP2c, which would argue against a critical role for apoE in cytoskeletal maintenance. However, results from the in vitro studies show that the formation of apoE–tau complexes can be detected at very low concentrations of apoE [10^{-9} M (106)], and, furthermore, evidence seems to point to higher levels of apoE in human neurones (see the next section). The relevance of apoE–tau interactions to normal physiology, or indeed AD, is far from clear at present. In addition to experiments to study apoE function in vivo, it will be important to confirm the correlation between buildup of neurofibrillary tangles and APOE4 genotype, as has been described by Ohm et al. (65).

Regulation of Neurite Outgrowth

The first study to demonstrate a difference in the activity of different apoE isoforms on intact neurones was reported by Nathan et al. (61). Treatment of rabbit dorsal root ganglion neurones with apoE3 resulted in an increase in neurite extension and a decrease in neurite branching. In contrast, apoE4 treatment reduced both neurite extension and neurite branching. Furthermore, the effects of apoE4 predominated over apoE3 when combinations of the two isoforms were employed (61). These effects of the different apoE isoforms were dependent upon the presence of very low density lipoprotein (VLDL), since neither apoE3 nor apoE4 had any effect alone, indicating that apoE needs to be presented to neurones as a lipoprotein complex to elicit these effects. These effects were subsequently reproduced with mouse neuroblastoma (Neuro-2a) cells stably expressing human apoE3 or apoE4 (5). The differential effects of the apoE isoforms were again dependent on the presence of VLDL and were blocked by agents preventing the uptake of lipoproteins by LRP (5,34).

Further studies of Neuro-2a cells treated with the different apoE isoforms demonstrated that apoE4 treatment resulted in production of fewer microtubules and a greatly reduced ratio of polymerised to monomeric tubulin than treatment with apoE3 (62). It remains to be established whether apoE exerts its effects on the neuronal microtubular cytoskeleton by a direct or an indirect mechanism. However, the demonstration of a differential direct interaction between apoE and the microtubule associated proteins MAP2 and tau (106) does define a novel mechanistic basis for the differences observed in cell cultures treated with the different isoforms.

An alternative mechanism by which apoE might affect neuronal growth and plasticity is interaction with extracellular matrix proteins. It has been shown that apoE can interact with extracellular matrix proteins such as laminin (38), and experiments in which neurones were cultured on substrates of either laminin alone or laminin plus apoE showed increased cell number and neurite outgrowth in the presence of ApoE. However, unlike the isoform-specific effects of apoE in the Nathan (61) study, the effect in the presence of laminin was identical whether apoE3 or apoE4 was used.

With respect to the association of apoE4 with late onset Alzheimer's disease, the apoE4 isoform may contribute to development of the disease by inhibiting neuronal remodelling, as reflected by an inhibition of neurite extension in culture. In contrast, the enhancement of neurite extension in culture by apoE3 may reflect the ability of this isoform to promote remodelling of neuronal processes to compensate for the dendritic pruning observed in ageing and Alzheimer's disease.

Lipid Transport

The accepted role for apoE in the periphery is the mediation of lipid transport (47). ApoE3 and apoE2 tend to associate with high density lipoprotein (HDL) particles, whereas apoE4 tends to bind VLDL particles. The apoE–phospholipid complexes bind lipoprotein receptors and are internalised. ApoE3 and apoE4 bind equally well to the LDL and LRP receptors, whereas apoE2 binds with 100-fold lower affinity [reviewed by Mahley (47)]. Thus, APOE2 homozygotes show elevated levels of plasma cholesterol and have a high risk of atherosclerosis [see (93) for review].

Because interaction of apoE with the LDL and LRP receptors is identical for the APOE3 and APOE4 isoforms, such interaction cannot account for the association of the latter with AD. However, the intracellular fate of apoE–lipid complexes may differ between apoE3 and apoE4, as discussed earlier. Although relatively few studies have examined the role of apoE in lipid transport in the CNS, the high lipid content of the brain (>50%) and the restricted expression of members of the apolipoprotein family would likely favour as important a lipid-transport role for apoE in the CNS, as in the periphery. Furthermore, experimental models of brain injury have shown that lipid transport plays a key role in subsequent synaptic regeneration and that this process occurs concurrently with increased expression of apoE (see later). Aspects of lipid trafficking by apoE may also account for some of the in vitro interactions described elsewhere in this review. For example, in the studies of the effects of apoE on neurite outgrowth described above (61), beta-VLDL was required for the effect.

Interaction with β-amyloid

The deposition of dense plaques of β-amyloid is a necessary pathologic marker of Alzheimer's disease. However, there is considerable diversity of opinion regarding the significance of plaque formation to the etiology of the disease. Despite this, it has been an important objective to demonstrate or refute a link between β-amyloid and apoE. Increased deposition of β-amyloid has been observed in the cortex of AD patients who carried an APOE4 allelle compared with those who did not (20,65,87). However, no increase in the rate of progression of the disease was reported (87). It is curious that in Down's syndrome, a condition that shows many of the pathologic traits of AD, no increase in amyloid load was noted in the brains of APOE4 carriers, although in this case, the APOE4 genotype was associated with earlier death (48). This inconsistency may highlight the problems of extrapolating the rate of progression of a disease from pathologic markers identified postmortem. Increased deposition of β-amyloid in APOE4 carriers has also been documented in patients who have died of head injuries or stroke (64). This implies that apoE may have a more general role in the response to brain injury rather than a specific one relating to AD [see (89)]. However, this would be consistent with apoE as a risk factor for AD rather than a cause.

ApoE has been shown to be associated with β-amyloid plaques in the AD brain (Fig. 2), although it should be noted that other members of the apolipoprotein family, apoJ and apoA1, were also present (30), which is suggestive of a simple aggregation of lipophilic proteins rather than a specific interaction. However, a number of groups have studied the interaction between apoE and β-amyloid. Strittmatter et al. (107) have shown that human apoE from plasma could bind β-amyloid under cell-free conditions. They showed that the

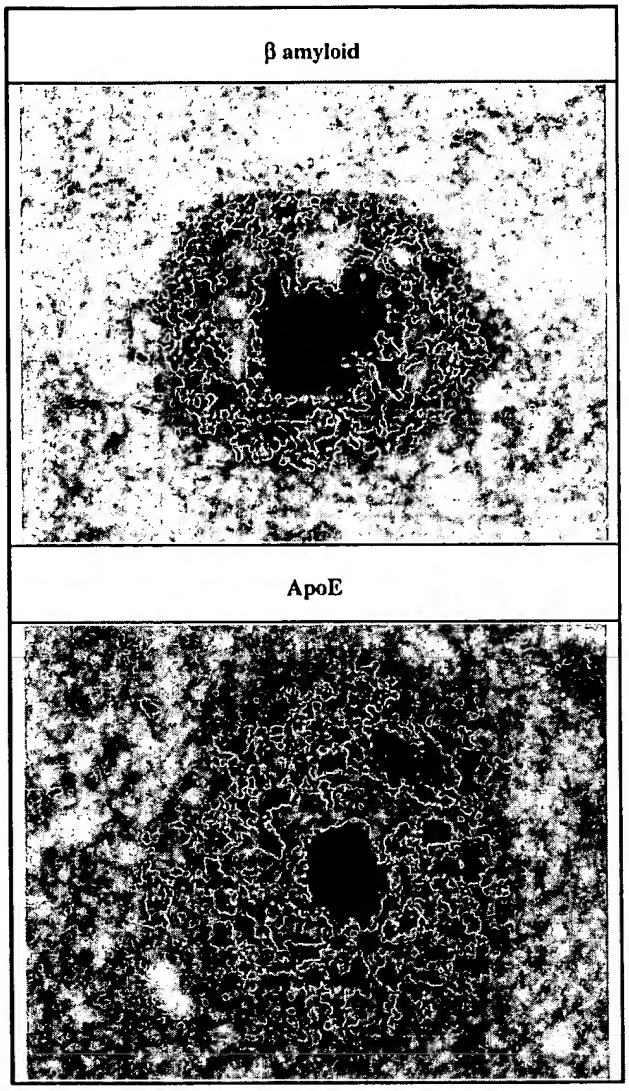

FIG. 2. Adjacent sections of postmortem temporal cortex tissue from an 88-year-old female AD patient. One section was immunostained with an antibody to β-amyloid (10 μg/ml; cat. no. 1381431, Boehringer), and an adjacent section was stained with an antibody to apoE (1:8000; cat. no. AB947, Chemicon). Note the close overlap between the distribution of the two proteins within the neuritic plaque. (Figure provided courtesy of Lorraine Fellows, Glaxo Wellcome, Stevenage.)

interaction may be dependent upon the oxidation state of apoE, since no interaction was seen in oxygen-free medium; furthermore, they found that apoE4 bound far more rapidly to β-amyloid than did apoE3. They suggested that oxidation of apoE could occur under inflammatory conditions around plaques in the AD brain, and that apoE might enhance the aggregation of amyloid. A similar interaction between apoE and β-amyloid was also shown by Chan et al. (12) using recombinant human apoE derived from *Escherichia coli*. However, in this study no difference was observed between apoE3 and apoE4. In contrast to both of these studies, experiments in COS cells in which C-terminal fragments of APP were overexpressed with apoE showed that apoE actually reduced the formation of amyloid aggregates (66). The authors saw no difference between apoE3 and apoE4, and concluded that apoE might inhibit amyloid fibril formation in vivo in an isoform-independent manner. A more detailed study by Evans et al. (18) found that apoE3, at physiological concentrations, could inhibit amyloid aggregation, with apoE4 showing a similar but less potent effect. Evans et al. proposed that the linkage between the APOE4 allele and AD was due to the reduced ability of APOE4 homozygotes to suppress amyloid formation. Some resolution of the inconsistency of the studies of apoE–amyloid interactions may come from a study by LaDu et al. (45), in which they showed that purification of human plasma apoE, which would denature and delipidate the protein, reduced differences between apoE3 and apoE4. Thus, those studies that have used purified preparations (12,66) may produce results that do not reflect the likely interactions of apoE in vivo.

The conformation of β-amyloid may also be a critical factor in the interaction with apoE and may account for some of the discrepancies of the in vitro studies. Transformation of β-amyloid from an α-helical soluble structure to an aggregated β-pleated sheet is the defining step in amyloid fibril formation. ApoE binds more readily to the β-pleated sheet form (24). Human plasma apoE was found to increase the aggregation of β-amyloid in vitro, as might be expected from the studies of Strittmatter et al. (105) and Chan et al. (12). Significantly, however, apoE was able to effect a conformational change of β-amyloid from the α-helical form to the β-pleated sheet form, as measured by circular dichroism, under conditions that minimised aggregation of the peptide (100). Thus, the authors concluded that apoE actively enhanced β-amyloid aggregation, although they were unable to distinguish between the different isoforms of apoE.

One interesting hypothesis regarding the interaction between apoE and β-amyloid is that the former may actively scavenge amyloid from the extracellular space and thus prevent its aggregation. Significantly, active microglia, some of which were immunoreactive for apoE, have been observed in clusters close to the core of amyloid plaques (112). This hypothesis is consistent with recent studies of apoE knockout transgenic mice, in which Robertson et al. found early deposition of β-amyloid in the brains of the mice, suggestive of a deficit in their ability to clear extracellular amyloid (unpubl. report: Roses, pers. comm.). On the basis of this result, coupled with the increased amyloid load observed in APOE4-carrying AD patients, it is possible that possession of the APOE4 allele is equivalent to a loss of apoE function; therefore, reduced interaction with β-amyloid, such as that observed by Evans et al. (18) or Ohman et al. (66), might be more relevant to Alzheimer's disease. Further studies in transgenic animal models of AD to investigate the effects of different human apoE isoforms on amyloid load will be required to resolve these issues.

Contribution to Oxidative Stress

It has been suggested that the generation of free radicals may contribute to the neuronal damage seen in Alzheimer's disease (6,67). In a study of apoE-deficient mice, Matthews and Beal (56) observed increased levels of nitrotyrosine residues in various brain regions, which is indicative of heightened oxidative stress mediated by peroxynitrate. They proposed that a role for apoE in the CNS might be to scavenge reactive oxygen species. ApoE2 contains two cysteine residues at positions 112 and 158; the Cys 158 residue is mutated to arginine in

apoE3. ApoE4 contains no cysteine residues in these positions, being identical to E3 except in mutation of Cys to Arg at the 112 position. The cysteine residues may be readily oxidised and so may mop up free radicals. Thus, apoE2 would show greater scavenging ability than apoE3, whilst apoE4 would not show this activity and so would be more equivalent to the knockout. However, the hypothesis is at odds with theories to account for the difference in β-amyloid binding of apoE3 versus apoE4. In the latter case, the rate of binding has been shown to be dependent upon oxidation of apoE, and it is proposed that apoE4 is more rapidly oxidised than apoE3 (105). Whatever the relative abilities of apoE3 and apoE4 to neutralise free radicals, the 1:1 stoichiometry of this interaction and the low levels of apoE, at least in rodent neurones, would argue against such a role, although neuronal levels of apoE may increase dramatically in tissues undergoing trauma (see the next section). If the theory is correct, then one might expect to see apoE localised in regions of the cell where free radicals are likely to be generated, e.g., peroxisomes and mitochondria. However, more specialised mechanisms, such as the enzyme superoxide dismutase, exist to deal with free radicals in neurones. This is likely to be an area of considerable future interest, and the availability of human apoE isoform transgenic animals models is likely to be of benefit in this research.

IN VIVO STUDIES OF ApoE

In vivo studies on apoE have been somewhat limited to date and are focussed in two areas. The first involves the production and study of mice in which the APOE gene has been either eliminated or replaced by hAPOE isoforms [the mouse normally expresses a single apoE isoform most similar in structure to apoE4; see (8)]. The second involves the study of apoE expression in models of CNS trauma, notably cerebral ischaemia, and CNS remodelling after entorhinal cortex lesions.

Transgenic Research

A valuable means with which to investigate the function of genes and their protein products is the technique of gene targetting and the generation of transgenic animals overexpressing, or deficient in, the gene of interest. In 1992, two groups, Maeda and coworkers (University of North Carolina, Chapel Hill, NC) and Breslow and coworkers (Rockefeller University, New York), reported the generation of APOE knockout mice by homologous recombination to disrupt the APOE gene in an ES cell line (129 or E14 derived) (70,71). The resulting chimeras were backcrossed to either 129, BALB/cJ, or C57/B6 lines. As might be expected, the major phenotype of these animals was high plasma triglyceride and cholesterol levels, the latter being approximately five times the level of wild-type controls (118). This underlies the development of fatty streaks and foam cell deposits in the aortic sinus, ultimately leading to complex atherosclerotic lesions virtually indistinguishable from those seen in humans (71,94,118). These may begin to develop in the knockout mice as early as 3 months of age (71,118).

In terms of the effect of APOE gene deletion on CNS morphology and function, the most comprehensive analysis to date has been reported by Masliah and coworkers (53), primarily using the Rockefeller-derived stock (71). They reported significant reductions in dendritic and synaptic density in hippocampal and cortical areas, respectively, measured using MAP2 and synaptophysin immunoreactivity. Further changes in other cytoskeletal markers, e.g., α- and β-tubulin, were also noted (53) and may be seen to be consistent with the belief that apoE may be involved in the stabilisation of microtubule assembly [see (103,104,106)]. These changes became apparent as early as 4–8 months of age although, perhaps surprisingly, they did not increase in magnitude beyond this period (53). The synaptophysin results have not been replicated in a very preliminary study reported by Gandy et al. (19) using mice derived from the same transgenic line as that used by Masliah, hence further studies are necessary. More recently, Michaelson and colleagues have published reports suggesting cholinergic deficits and increased AT8 and ALZ50 immunoreactivity of apoE knockout brain suggestive of tau hyperphosphorylation [(21,25); although see (58)]. These latter findings are of interest given the proposal that, in humans, to account for the association between the E4 genotype and AD, there may be isoform differences in tau binding [see (106)].

Markers of oxidative stress include increases in lipid peroxidation and nitration of proteins [e.g., (6)]. Matthews and Beal (56), reported that apoE knockout mice show increased protein nitration, assessed by measuring 3-nitrotyrosine levels by HPLC, indicating formation of peroxynitrate, a highly reactive and neurotoxic free radical (4,113). These CNS changes are largely non-region-specific and do not extend to markers of lipid peroxidation, measured by DHBA assay. It is of obvious interest to explore these observations further and to examine a potential relationship between protein nitration and any synaptic disruption, as reported by Masliah and coworkers (53).

Given these reported ultrastructural and biochemical differences between apoE knockouts and their wild-type controls, one might predict behavioural/cognitive differences, particularly because much of this pathology has been reported in limbic regions, e.g., the hippocampus and frontoparietal cortex. The only full report, albeit brief, that addresses this issue, suggests that knockout mice may show cognitive impairments, as assessed using a modified water maze paradigm designed to measure working memory by two-trial acquisition to a hidden island, the location of which is changed daily (25). Curiously, despite showing poor learning within days (working memory), the knockouts showed good learning across days (reference memory), the magnitude of which seemed superior to controls. We are presently addressing a number of these issues, including the behavioural/cognitive differences between apoE knockouts and their wild-type littermate controls. Initial studies have failed to reveal any differences (see Fig. 3), although it must be noted that these experiments have only been conducted in 2-month-old mice. The data reported in the studies of Masliah et al. (53), Gordon et al. (25), and Genis et al. (21) were in mice at least 4–8 months of age. We are presently allowing a separate colony of these animals to age before further behavioural/ultrastructural studies are undertaken.

An important point to note about transgenic research, particularly in behavioural analyses (but presumably in morphological/biochemical studies as well), is the nature of the genetic background of the transgenic animal under study. Most gene targetting is carried out in ES stem cells derived from the mouse strain 129. Because of inherent breeding difficulties with the 129 strain, frequently these ES cells are introduced into a second strain, e.g., C57/B6. The resulting offspring are thus a complex mix of background genes from either strain, in addition to the gene target. It is now becoming evident that mouse strains may show wide variations in motoric/cognitive functions, with 129 and C57/B6 strains being extreme examples

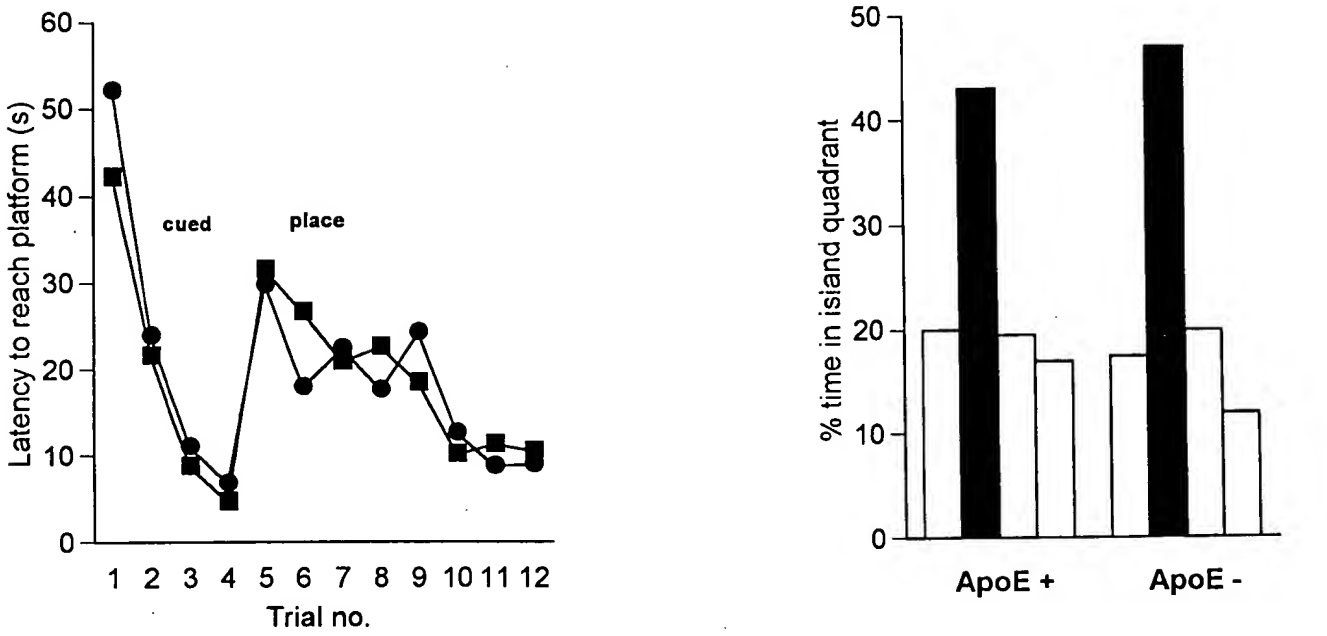

FIG. 3. Effect of APOE gene deletion on mouse cognitive behaviour assessed using the Morris water maze. Mice (n = 12 per group) were trained to locate a visible platform of variable location (cued task) followed by a hidden platform of fixed location (place task). This was followed by a 60-s probe task, in which the platform was removed and the spatial bias shown by the animal for the island quadrant (filled bar) was recorded. Note that there was no difference between APOE knockout (ApoE−, ■) and wild-type littermate controls (ApoE+, ●), both of which showed similar cued and place learning. The APOE genotype was subsequently confirmed by measurement of plasma cholesterol levels (ApoE+, 2.6 mmol/litre; ApoE−, 12.6 mmol/litre). (Figure adapted from data provided by Dr. Richard Anderson, Glaxo Wellcome, Stevenage.)

(22,116). Consequently, it has recently been suggested that inadequate backcrossing may lead to transgenics whose reported phenotypical differences may be more attributable to background genes rather than to the gene target (22). It is therefore imperative that, in transgenic research, variability of genetic background is minimised by repeated backcrossing, use of littermate wild-type controls, or use of rescue experiments as a control where the phenotypical difference between a transgenic and its control is eliminated by replacement of the missing functional protein (22). In our present studies with the apoE knockout mice, we are using animals backcrossed at least six times to the C57/B6 strain, as well as wild-type littermate controls.

A further issue of potential importance that has not been addressed to date in the apoE transgenic studies is the development of atherosclerotic plaques in the knockout animals and the potential for compromised cardiovascular function that may contribute to the CNS pathology. There are a few points to note. First, longitudinal studies are unable to dissociate these events, as each may develop in mice as early as 3–4 months of age (53,118). Heterozygous apoE-deficient mice, which have half the normal apoE gene dosage and develop less extensive atherosclerosic lesions, show little or no evidence of CNS pathology (53). Finally, the accelerated atherosclerosis results in increased plasma lipid peroxidation and oxidative stress (32). It remains to be seen whether this could lead to the CNS changes reported by Matthews and Beal (56). Therefore, there is presently no evidence to eliminate this potential association. The ideal tool for this research would be an animal with normalised apoE function in the periphery but still deficient in the CNS. An elegant approach to this has been reported by Linton et al. (46), who described a technique in which apoE knockouts received bone marrow transplantation of macrophage cells (which synthesise apoE) from wild-type mice. These animals demonstrated virtually complete absence of atherosclerosis and normalised blood cholesterol levels (46), yet presumably retained no central apoE expression.

Clearly of more fundamental interest to apoE research in relation to AD is the generation of mice expressing the different human apoE isoforms in the absence of endogenous mouse apoE expression. Such transgenics have recently been produced (117). A number of lines of varying apoE gene copy number have been made, and in each (except two apoE2 lines) the elevated plasma cholesterol seen in the knockout animals has been substantially corrected, confirming functional expression of the transgene. In these animals, the apoE gene is under the control of the human promoter, since constructs used to make the transgenics consisted of human genomic DNA fragments containing regulatory sequences flanking both ends of the apoE gene (117). Perhaps a feature of such human regulatory sequences is the neuronal localisation of apoE in the CNS of these transgenics [similar to human and primate; see (28,29,88)] compared with normal mice, where apoE localisation is largely restricted to astrocytes. Clearly, these animals will represent useful tools with which to study apoE isoform differences in basic brain biology and function, and in models of CNS injury and repair. This work may also raise important species differences between the expression patterns of apoE, and suggest that nontransgenic rodents may be inappropriate models for the study of the role of apoE in humans.

ApoE in CNS Injury and Repair

It is the work of Judes Poirier and colleagues that has made the greatest contribution to the study of apoE and CNS repair.

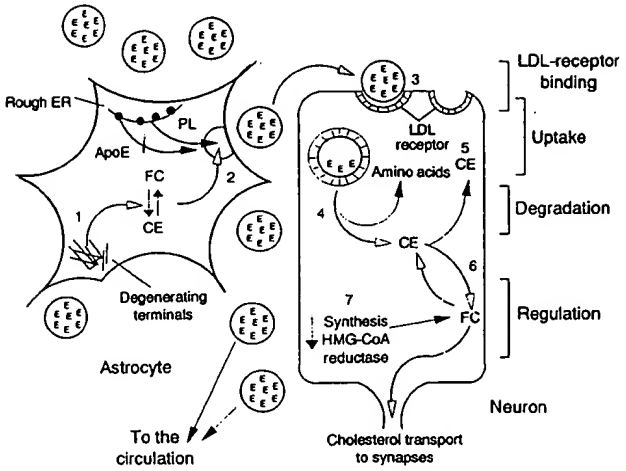

FIG. 4. Schematic representation of hypothesised cholesterol–phospholipid recycling process in the dentate gyrus region following an entorhinal cortex lesion (based on the work of Poirier and coworkers; see the section on *ApoE in CNS Injury and Repair*). Degenerating nerve terminals are internalised and degraded within astrocytes (**1**). Cholesterol is liberated and may combine with newly synthesised apoE to form apoE–cholesterol–lipoprotein complexes (**2**). These apoE complexes are then directed into the circulation, after which they may be taken up into brain cells requiring lipids, through uptake via the LDL, or LRP (or both) receptor pathway (**3**). Once internalised, the cholesterol is released (**4**) for dendritic growth or synaptogenesis. As a result of this internalisation, neuronal cholesterol synthesis is repressed via reduced synthesis of HMG-CoA reductase (**7**). E, apoE; PL, phospholipids; CE, cholesterol esters; FC, free cholesterol. [Figure reproduced from reference (72) with permission of the author.]

This work has used a model system in rats of entorhinal cortex lesions (ECL) that produce a well documented synaptic loss in the denervated dentate gyrus region followed by compensatory resprouting from local septohippocampal neurones and contralateral entorhinal cortex afferents (51,55,101,102). A series of experiments initially revealed a marked elevation of several RNA transcripts following ECL, one identified as GFAP (77) and another encoding a 35-kDa polypeptide subsequently identified as apoE (75). In situ hybridisation showed the site of apoE synthesis to be reactive astrocytes (GFAP double labelling) in the denervated dentate gyrus (76). Further experiments studied the relationship between apoE expression and cholesterol uptake/biosynthesis and eventually led to the scheme summarised in Fig. 4. Essentially, cholesterol released during terminal degeneration following lesioning is taken up by astrocytes that synthesise apoE. It is hypothesised that stable apoE–cholesterol–lipoprotein complexes are formed, released, and targeted to local neurones undergoing axonal growth or synaptogenesis by uptake via apoE receptors [LRP, LDL, or VLDL; see (10,41,79,108,115)], although Poirier has shown that the LDL receptor is upregulated in response to the lesion (72,73). Thus, it is suggested that apoE serves as a transporter, carrying lipids between astrocytes and sprouting neuronal targets. We have recently undertaken similar experiments in nontransgenic C57/B6 mice and found the expression of apoE to be limited to hippocampal regions undergoing synaptogenesis and to precede this process (33), consistent with the work of Poirier and coworkers.

Considering this fundamentally important role for apoE, it is not surprising that processes of synaptic repair appear to be compromised in apoE-deficient mice after ECL (52). However, following sciatic nerve crush, apoE levels increase dramatically in genetically unmodified animals (40,47), but recovery still persists in apoE knockouts and indeed is indistinguishable from controls (78). It is believed that in the periphery, unlike the CNS, alternative apolipoproteins may substitute for apoE, e.g., apoD, apoA-I, and apoA-IV.

A number of studies have examined the pattern of apoE expression following transient cerebral ischaemia in the rat and the gerbil (26,35,42,44). Each reports a significant increase in apoE immunoreactivity, notably in neurones within the CA1/CA2 field, the cell population particularly vulnerable to neurodegeneration. In situ hybridisation shows a change in apoE mRNA to parallel that of GFAP, suggesting that apoE is synthesised by astrocytes (2). It remains contentious as to whether neurones actually synthesise apoE or, alternatively, if apoE is exclusively derived from astrocytes and taken up into neurones via LDL or LRP receptors (see earlier sections). Interestingly, the peak of apoE expression coincided with the period of cell death, leading to the question of whether apoE is expressed in dying CA1/2 neurones as a response that enhances survival, or whether it contributes to the cell death.

If it is to be believed that apoE has a more general role in the CNS response to injury, then it might be predicted that the APOE genotype may influence the onset or course of other neurodegenerative diseases or acute CNS trauma. There seems little evidence to suggest that the APOE genotype is linked to Parkinson's disease (49), amyotrophic lateral sclerosis [motorneurone disease (59)], Down's syndrome (39,48,50), or hippocampal sclerosis dementia (111). However, preliminary reports have suggested an association between the APOE4 genotype and poorer outcome following intracerebral haemorrhage, as assessed using the Barthel diagnostic index (1). Additionally, the APOE4 allele may also serve as a risk factor in recovery from head trauma (64,99). Furthermore, recovery following coronary bypass surgery is influenced by a variety of factors, including APOE genotype (63). This is clearly an area of considerable interest, and it is likely that in the future a number of similar epidemiological studies should help to clarify the issue.

ApoE and the Cholinergic System

A possible association between ApoE genotype and cholinergic dysfunction in AD has been proposed, based on postmortem examination of brain tissue. Thus, with increasing APOE4 allele copy number, there is a reduction in hippocampal and cortical cholinacetyltransferase (ChAT) levels and reduced numbers of acetylcholinesterase-positive neurones in cholinergic cell body regions, including the diagonal band of Broca and nucleus basalis of Meynert (72,74). Riekkinen and coworkers have also essentially replicated these findings (96). It has been proposed that this apparent sensitivity of the cholinergic system to the APOE genotype may be linked to its reliance on phospholipid metabolism (72,74). It is interesting to note that Gordon et al. (25) reported that mice deficient in apoE to show evidence for a central cholinergic hypofunction, although we have failed to replicate this observation (Anderson et al., unpubl. work).

The cholinesterase inhibitor tacrine (Cognex®) is presently the only drug available for the treatment of AD in the USA. There have been a number of clinical studies to evaluate its efficacy in AD, and each suggests considerable variation in patient response to this drug. Recently, Poirier and colleagues have suggested that part of this variability may be due to the APOE genotype, with patients carrying one or two copies of the E4 allele being poor responders (74), the explanation being

that these individuals show greater atrophy of the cholinergic system. An alternative explanation is misdiagnosis: on post-mortem examination, approximately 30% of non-E4 patients diagnosed with probable AD show incorrect diagnosis, for they display pathology typical of other dementias such as Pick's disease, hippocampal sclerosis dementia (83). In contrast, E4 carriers diagnosed with probable AD show about 98–100% accuracy of diagnosis (83). Hence, tacrine may be more beneficial in the non-AD cases. Future studies with newer cholinomimetics will undoubtedly establish the robustness of these associations. In any case, these data highlight an issue that is likely to become increasingly important in clinical trial design: that is, an individual's genotype may influence treatment response and in future may determine the treatment strategy being used.

CONCLUSION

The fundamental question concerning the role of apoE in the CNS is whether it serves a physiological and/or pathologic role. Studies of the expression of apoE in disease tissue, including AD brain, necessarily address a pathologic role for the protein; similarly, studies of apoE in CNS injury and repair provide evidence that the protein is recruited to stressed or dying neurones, either to support regeneration or to oversee their demise. In this respect, epidemiological studies designed to clarify the influence of the APOE genotype on other neurodegenerative conditions will be of interest.

The influence of the APOE genotype on Alzheimer's disease is clearly demonstrated. However, the question remains, does apoE contribute to the onset and early stages of disease, or does it simply affect the progression of the disease? Association of apoE with diverse pathologic states would seem to point to the latter. However, recent data from PET studies showing abnormal glucose metabolism in the cortex of APOE4 homozygotes up to 20 years before they develop symptoms of disease (80) are the first indication that the protein may be involved early in the disease process. This is supported by observations that in a nondemented aged population, persons carrying at least one APOE4 allele had poorer cognitive test scores and smaller hippocampal volume (97).

At the preclinical research level, there is a clear need for further study of potential roles for apoE in the CNS, such as lipid transport, as an antioxidant, or regulation of neuronal cytoskeleton. These lines of investigation are presently quite controversial, but will benefit from a better understanding of the expression and localisation of apoE in neurones and glia. Transgenic studies are still in their infancy, but the availability of different lines harbouring human apoE isoforms (117) will doubtless prove a valuable means to investigate isoform differences in both physiological and pathologic functions of apoE.

These are early days for apoE research, although there has been rapid progress driven largely by the recent estimates that approximately 60–70% of all AD cases are linked to the APOE4 genotype. There is considerable optimism that an understanding of how apoE-isoform differences can modify the risk of AD will eventually lead to an effective treatment for this devastating disease.

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Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group.

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OBJECTIVE: To determine the influence of heterozygosity for the delta 32 mutant CCR-5 allele on HIV-1 disease progression. DESIGN: HIV-1 disease progression and serum viral load were analysed according to the C-C chemokine receptor (CCR)-5 genotype in 412 Caucasian patients (319 men and 93 women) with a known date of seroconversion, who were enrolled in the SEROCO cohort (median follow-up, 74 months). RESULTS: The frequency of heterozygosity for the mutant allele was 17% and did not differ according to sex or risk factor of HIV infection. Heterozygotes were significantly less likely than patients with two functional alleles to have symptomatic primary infection. Their serum viral load was lower during the 6- to 24-month plateau phase after seroconversion. This difference persisted afterwards, although the rate of decline in CD4+ cells was similar. Kaplan-Meier survival curves showed slower progression to clinical AIDS in heterozygotes during the first 7 years following infection ($P < 0.02$), the two curves tending to join thereafter (overall log-rank test, $P = 0.17$). However, the interaction term with time did not reach significance in a Cox model. The overall relative risk of progression was 0.67 (95% confidence interval, 0.38-1.18) and was not influenced by adjustment for age at seroconversion or symptomatic primary infection. After adjustment for early viral load the relative risk was 0.83. Pneumocystis carinii pneumonia and toxoplasmosis were less likely to be the first AIDS-defining illness in heterozygotes than in the other patients (0 versus 24.7% of AIDS cases, $P = 0.04$), despite similar management. CONCLUSION: Deletion of one CCR-5 gene allele appears to protect against HIV-1 disease progression, mainly during the early years of the infection. Heterozygosity for the deletion leads to persistently lower viral load, and also seems to protect against some opportunistic infections.

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Evidence for a Major Quantitative Trait Locus on Chromosome 17q21 Affecting Low-Density Lipoprotein Peak Particle Diameter

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Background—Several lines of evidence suggest that small dense LDL particles are associated with the risk of coronary heart disease. Heritability and segregation studies suggest that LDL particle size is characterized by a large genetic contribution and the presence of a putative major genetic locus. However, association and linkage analyses have thus far been inconclusive in identifying the underlying gene(s).

Methods and Results—An autosomal genome-wide scan for LDL peak particle diameter (LDL-PPD) was performed in the Québec Family Study. A total of 442 markers were genotyped, with an average intermarker distance of 7.2 cM. LDL-PPD was measured by gradient gel electrophoresis in 681 subjects from 236 nuclear families. Linkage was tested by both sib-pair–based and variance components–based linkage methods. The strongest evidence of linkage was found on chromosome 17q21.33 at marker D17S1301, with an LOD score of 6.76 by the variance-components method for the phenotype adjusted for age, body mass index, and triglyceride levels. Similar results were obtained with the sib-pair method ($P<0.0001$). Other chromosomal regions harboring markers with highly suggestive evidence of linkage ($P\leq0.0023$; LOD ≥1.75) include 1p31, 2q33.2, 4p15.2, 5q12.3, and 14q31. Several candidate genes are localized under the peak linkages, including apolipoprotein H on chromosome 17q, the apolipoprotein E receptor 2, and members of the phospholipase A_2 family on chromosome 1p as well as HMG-CoA reductase on chromosome 5q.

Conclusions—This genome-wide scan for LDL-PPD indicates the presence of a major quantitative trait locus located on chromosome 17q and others interesting loci influencing the phenotype. (***Circulation.*** **2003;107:2161-2368.)**

Key Words: genome ■ lipoproteins ■ genetics ■ genes

A number of case-control as well as prospective studies reveal an increased risk of coronary heart disease in patients with small dense LDL compared with those having larger, more buoyant LDL particles.[1] Heritability studies based on twins suggested that approximately one third to one half of the variation in the LDL peak particle size can be attributed to genetic influences.[2,3] Complex segregation analyses of small dense LDL phenotypes have been performed with data from different types of family structures, different criteria for proband ascertainment, and the use of different techniques to characterize LDL heterogeneity.[4–9] Nevertheless, the model providing the best fit to the data included either a dominant, a recessive, or an undetermined mode of inheritance for the trait. Furthermore, the allele frequency determining the small dense LDL phenotype ranges from 19% to 42%, with reduced penetrances in young men and premenopausal women. However, these studies unanimously provided evidence in favor of a gene with a major effect on LDL particle phenotypes.

Association studies with candidate genes have been inconsistent in finding genes associated with small dense LDL. The −250G→A polymorphism within the hepatic lipase promoter was associated with buoyant LDL particles.[10] However, the −514C→T polymorphism, which is in complete linkage disequilibrium with the −250G→A polymorphism,[11] showed no effect on LDL particle size.[12,13] The apolipoprotein (apo) E genotype was also associated with the small dense LDL phenotype. However, some studies have reported smaller particles for subjects carrying the E4 allele,[14–16] and other studies did for subjects carrying the E2 allele.[17,18] In contrast, others have shown that LDL particle size did not differ among the apoE genotypes.[19] Additional candidate

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TABLE 1. Descriptive Statistics of LDL Peak Particle Diameter and Covariates in Each of the Sex and Generation Groups

Variables	Fathers (n=137)	Mothers (n=194)	Sons (n=148)	Daughters (n=202)
LDL-PPD, Å	261.2±5.4	264.3±5.2	262.6±4.5	264.4±4.5
Age, y	55.5±9.2	55.5±12.7	26.6±10.0	28.2±10.7
BMI, kg/m^2	28.9±6.3	28.7±8.0	26.5±6.9	27.6±8.7
Triglyceride, mmol/L	1.98±1.23	1.79±2.40	1.35±0.71	1.27±0.59

Values are mean±SD.

genes, including cholesteryl ester transfer protein,[20] microsomal triglyceride transfer protein,[21] cholesterol 7α-hydroxylase,[22] apoB-100,[23] apoC-III,[24] and ACE[16] were investigated for potential effects on small dense LDL phenotypes. These studies revealed either the absence of an association or the presence of an association only in particular subgroups.

Results from linkage studies are equivocal. After linkage of small dense LDL with the apoB (the protein moiety of LDL) gene locus on chromosome 2 was excluded,[25,26] suggestive linkage to the LDL receptor locus on chromosome 19 has been reported.[27,28] However, subsequent sequencing of the entire coding regions of the LDL-receptor gene did not reveal any sequence variants, thus weakening the hypothesis that a mutant LDL-receptor allele is responsible for the dense LDL phenotype.[29] Other candidate loci, including hepatic lipase,[12] lipoprotein lipase,[30] cholesteryl ester transfer protein,[28,31,32] apoA1-CIII-AIV complex,[28,32] and the manganese superoxide dismutase,[28,32] have been shown to be linked with the small dense LDL phenotype. Unfortunately, most of these linkages have not been replicated.[33,34] On the basis of these results, Austin et al[34] emphasized the necessity of finding new genetic loci, other than those harboring known candidate genes, to identify the genes potentially involved in determining the small dense LDL phenotype. Genome-wide scans are particularly suited for this purpose. Previous genome-wide scans have focused on variation in cholesterol concentrations of LDL size fractions. Rainwater et al[35] found 2 quantitative trait loci (QTLs) on chromosome 3 and 4 with logarithm of the odds (LOD) scores >3 for LDL size fraction 3 (LDL-3), a fraction that contains small LDL particles. This study demonstrates the existence of QTLs affecting the concentration of cholesterol within a particular subpopulation of LDL but do not provide evidence of QTLs responsible for the size of the LDL particle by itself. To the best of our knowledge, the only whole-genome scans on LDL particle size were performed on 240 individuals ascertained through 18 unrelated familial combined hyperlipidemic probands.[12] Results suggest a locus over the hepatic lipase gene on chromosome 15 with a LOD score of 2.2. Here, we report the results of an autosomal genomic scan for LDL peak particle diameter (LDL-PPD) measured by gradient gel electrophoresis.

Methods

Population

The Québec Family Study is an ongoing project composed of French-Canadian families that has been described previously.[36] In the present study, a total of 681 subjects from 236 nuclear families had available data on LDL-PPD. Table 1 presents the characteristics of subjects in each of the sex and generation groups. The Laval University Medical Ethics Committee approved the study, and all subjects provided written informed consent. All the procedures followed were in accordance with institutional guidelines.

Phenotype

LDL-PPD was measured by gradient gel electrophoresis from plasma obtained after a 12-hour fast. Details on the technique have been provided previously.[37]

Genotypes

Genomic DNA was prepared by the proteinase K and phenol/chloroform technique. DNA preparation, polymerase chain reaction conditions, and genotyping are described in detail elsewhere.[38] Genotypes for each marker were typed by use of automatic DNA sequencers and the computer software SAGA from LICOR. The results were stored in a local dBase IV database, GENEMARK, which inspects results for mendelian inheritance incompatibilities within nuclear families and extended pedigrees. A total of 335 microsatellite markers (dinucleotide, trinucleotide, and tetranucleotide repeats) selected from different sources, but primarily from the Marshfield panel version 8a, were available for this genome scan. The location of markers on the chromosomes in centimorgans (cM) were taken from version 9.0 of the Marshfield Institute map[39] and the Location Database map.[40] In addition, 107 polymorphisms in 63 candidate genes were included. The average intermarker distance for the whole set of 442 markers was 7.2 cM. The Genome Database[41] and the OMIM gene map[42] were used to identify candidate genes.

Statistical Analyses

LDL-PPD was adjusted for covariates by use of a stepwise multiple regression procedure, retaining only terms that were significant at the 5% level. Regression parameters were estimated within 6 age-by-sex (<30, 30 to 50, and ≥50 years; male versus female) groups after exclusion of outliers (±4 SD), and residuals were computed for all subjects. Residual scores were then standardized to a mean of 0 and an SD of 1. LDL-PPDs were adjusted for 3 different sets of covariates: (1) age up to the cubic polynomial, (2) age and body mass index (BMI), and (3) age, BMI, and triglyceride levels. These adjustments gave 3 phenotypes, arbitrary called LDL-PPD1, LDL-PPD2, and LDL-PPD3, respectively. The phenotypes were adjusted by use of SAS software (version 8.02).

The search for linkage between the phenotypes and the genetic markers was performed by 2 different approaches. First, linkage was tested by the new Haseman-Elston regression-based method, which models the trait covariance between sib-pairs, instead of the squared sib-pair trait difference used in the original method. It regresses the mean-corrected trait cross product on the number of alleles shared identical by descent (IBD). Single-point and multipoint estimates of alleles shared IBD were generated with the GENIBD software, and linkage was tested with the SIBPAL2 software from the SAGE 4.0 statistical package (SAGE, 2001).[43] The maximum number of sib-pairs was 352. Linkage was also investigated by the variance components–based approach implemented in the quantitative transmission disequilibrium test (QTDT) computer software.[44] By this approach, the phenotypic covariance among members of a family is

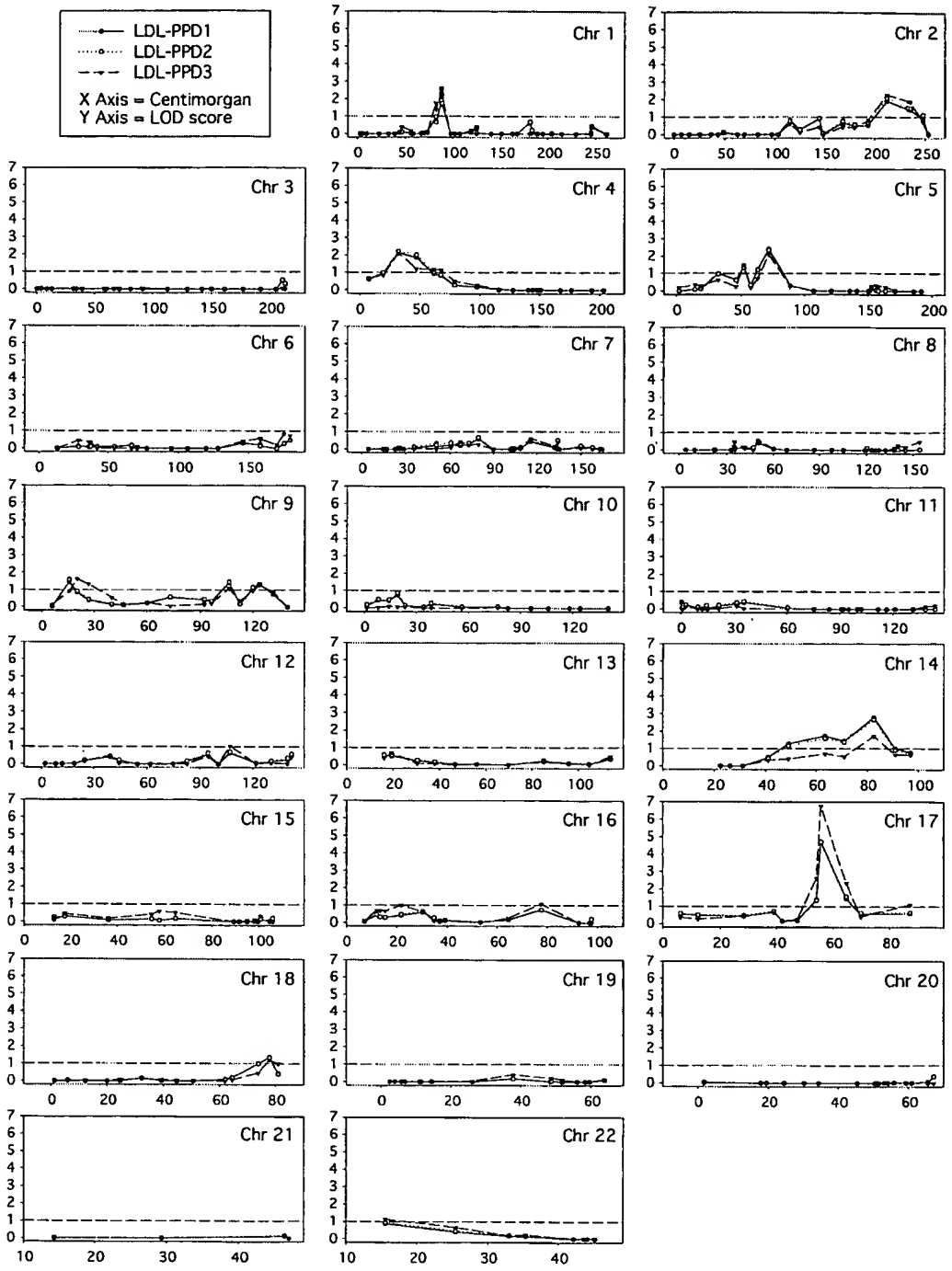

Figure 1. Quantitative transmission disequilibrium test linkage results for all autosomal chromosomes with LDL-PPD phenotypes. LOD scores are presented on *y* axis, and genetic distance is presented on *x* axis in centimorgans. LDL-PPD1, LDL-PPD2, and LDL-PPD3 indicate LDL-PPD adjusted for (1) age, (2) age and BMI, and (3) age, BMI, and triglyceride, respectively.

assumed to result from the additive effects of linkage caused by a major locus (a), a residual familial component resulting from polygenes (g), and a residual nonshared environmental component (e) that represents environmental effects unique to each family member. Linkage is tested by contrasting the null hypothesis of no linkage ($\sigma_a=0$) with the alternative hypothesis ($\sigma a \neq 0$) by use of a likelihood ratio test, as described previously.[45] The LOD score was computed as $\chi^2/(2 \log_e 10)$. The interpretation of linkage evidence was considered suggestive ($P \leq 0.01$; LOD ≥ 1.18), highly suggestive ($P \leq 0.0023$; LOD ≥ 1.75), or evidence of linkage ($P \leq 0.0001$; LOD ≥ 3.0).[46]

Results

An overview of the variance components–based linkage results for the 3 LDL-PPD phenotypes is given in Figure 1. Suggestive evidence of linkages ($P \leq 0.01$ or LOD scores ≥ 1.18 for at least one of the phenotypes) is summarized in Table 2. The strongest evidence of linkage, which was confirmed by both linkage methods, was found on chromosome 17q21.33. As shown in Figure 2, the peak linkages were found with marker D17S1301 for LDL-PPD1 (LOD=4.72),

TABLE 2. Results From the Genome Scan: Markers Showing Evidence of Linkage With the LDL-PPD Phenotypes According to the Linkage Methods Used

	LDL-PPD1			LDL-PPD2			LDL-PPD3		
Marker/Chromosome Location§/Distance, cM	*P*, Single Point	*P*, Multipoint	LOD Score	*P*, Single Point	*P*, Multipoint	LOD Score	*P*, Single Point	*P*, Multipoint	LOD Score
D1S203/1p31.1/82.250	0.19449	0.02915	0.97	0.20201	0.03117	0.98	0.17966	0.00777*	1.74*
D1S220/1p31.1/82.496	0.21395	0.11233	0.67	0.24852	0.12599	0.66	0.07821	0.02299	1.37*
LEPR/1p31/87.771	**0.000002‡**	**0.00037†**	**2.05†**	**0.000004‡**	**0.00046†**	**2.04†**	**0.00003‡**	**0.00009‡**	**2.56†**
D1S198/1p22.3/88.650	0.05148	0.00551*	1.70*	0.04796	0.00533*	1.74*	0.02932	0.00406*	2.21†
D2S1776/2q24.2/168.109	0.01446	0.00782*	0.66	0.01235	0.00639*	0.78	0.11668	0.07954	0.43
D2S1384/2q33.2/212.118	0.06188	0.00036†	1.91†	0.05694	0.00026†	2.11†	0.08574	0.00278*	2.27†
D2S434/2q36.1/233.148	0.02500	0.00708*	1.45*	0.03081	0.00878*	1.54*	0.01182	0.00873*	1.92†
IRS1/2q36.3/235.700	0.33181	0.00626*	1.39*	0.35506	0.00736*	1.49*	0.50910	0.01011	1.85†
D2S427/2q37.3/247.918	0.00374*	0.01128	0.93	0.00306*	0.00797*	1.12	0.02621	0.06192	0.74
D4S403/4p15.33/19.455	0.00888*	0.00669*	0.96	0.01018	0.00764*	0.99	0.02084	0.01663	0.83
D4S2397/4p15.2/32.246	0.03234	0.02079	2.14†	0.02866	0.01891	2.22†	0.04169	0.03278	2.11†
D4S1627/4p13/47.177	0.04631	0.01098	1.87†	0.04270	0.00960*	2.02†	0.09922	0.03438	1.21*
D4S3248/4q12/61.658	0.02666	0.01647	0.93	0.02682	0.01439	1.02	0.04046	0.00860*	1.18*
D4S3243/4q13.1/67.540	0.12255	0.02906	0.82	0.12301	0.02723	0.87	0.05296	0.00593*	1.15
D5S1986/5p13.3/32.470	0.10859	0.00258*	1.00	0.13063	0.00336*	0.97	0.30218	0.01240	0.63
D5S1470/5p12/46.124	0.00981*	0.01608	0.64	0.01003	0.01812	0.61	0.07421	0.07127	0.25
D5S1457/5p12/51.910	0.00890*	0.00149†	1.37*	0.00834*	0.00170†	1.35*	0.00126†	0.00042†	1.47*
CART/5q13-q14/63.001	0.70047	0.00558*	1.23*	0.68788	0.00549*	1.21*	0.77059	0.00778*	0.71
D5S1501/5q12.3/71.671	**0.000001‡**	**0.000004‡**	**2.40†**	**0.000001‡**	**0.000003‡**	**2.34†**	**0.000001‡**	**0.000008‡**	**2.10†**
ADRB2/5q31/156.383	0.01360	0.20977	0.05	0.01583	0.23011	0.04	0.00003‡	0.01212	0.33
TNFα/6p21.3/36.428	0.00488*	0.34210	0.08	0.00561*	0.35525	0.07	0.00023†	0.12755	0.35
TA184A08/6q24-q25.2/146.000	0.00300*	0.00304*	0.29	0.00309*	0.00299*	0.33	0.00845*	0.01232	0.41
D6S441/6q24.3/158.347	0.01456	0.03159	0.20	0.01611	0.03469	0.21	0.00572*	0.01122	0.55
D8S1110/8q11.1/50.229	0.01498	0.11057	0.41	0.01567	0.11257	0.44	0.00210†	0.06285	0.56
D9S925/9p22.3/15.738	0.00631*	0.00427*	1.46*	0.00420*	0.00295*	1.58*	0.06369	0.03619	0.90
D9S1121/9p22.1/20.396	0.10322	0.00627*	0.84	0.09028	0.00533*	0.91	0.02736	0.00210†	1.63*
D9S1118/9p21.1/26.815	0.32831	0.05855	0.39	0.31187	0.05127	0.44	0.14331	0.01152	1.32*
D9S938/9q31.1/106.261	0.03766	0.01388	1.43*	0.03897	0.01155	1.49*	0.04919	0.01748	1.08
D9S934/9q33.2/123.662	0.07024	0.01885	1.33*	0.07420	0.02006	1.35*	0.10882	0.01712	1.29*
D12S1045/12q24.33/142.100	0.00964*	0.02334	0.52	0.00867*	0.02157	0.58	0.02010	0.03435	0.40
D13S141/13q11/16.075	0.00326*	0.02313	0.54	0.00419*	0.02590	0.57	0.05748	0.10325	0.40
D13S787/13q12.11/19.370	0.66731	0.00435*	0.51	0.65641	0.00413*	0.56	0.50960	0.00491*	0.69
D14S587/14q21.3/49.255	0.00544*	0.01155	1.29*	0.00683*	0.01494	1.17	0.02507	0.06996	0.41
D14S592/14q23.2/63.513	0.01322	0.02929	1.73*	0.01486	0.03486	1.59*	0.02435	0.05471	0.71
D14S588/14q24.1/71.008	0.19152	0.06990	1.44*	0.17827	0.06968	1.38*	0.39519	0.14212	0.56
D14S53/14q31.1/82.701	**0.000008‡**	**0.00006‡**	**2.79†**	**0.000008‡**	**0.00009‡**	**2.65†**	0.000008‡	0.00067†	1.72*
D14S617/14q32.11/91.013	0.52394	0.00898*	1.01	0.53584	0.01267	0.91	0.57767	0.01788	0.66
D16S287/16p13.13/15.349	0.58176	0.02271	0.28	0.61340	0.02218	0.30	0.63070	0.00927*	0.66
D16S410/16p12.3/21.763	0.21593	0.00418*	0.41	0.20760	0.00375*	0.45	0.08646	0.00044†	1.00
D16S403/16p12.1/30.348	0.09036	0.00429*	0.57	0.08175	0.00346*	0.64	0.12627	0.00325*	0.62
D16S261/16q11.1/53.319	0.00144†	0.07305	0.02	0.00142†	0.07113	0.03	0.00012†	0.08361	0.02
D17S974/17p13.1/12.330	0.01033	0.04439	0.47	0.00956*	0.04380	0.50	0.07468	0.18376	0.27
D17S1290/17q21.32/54.190	0.00114†	0.00142†	1.39*	0.00167†	0.00184†	1.34*	**0.00110†**	**0.00042†**	**2.63†**

continued

TABLE 2. Continued

Marker/Chromosome Location§/Distance, cM	LDL-PPD1			LDL-PPD2			LDL-PPD3		
	P, Single Point	*P*, Multipoint	LOD Score	*P*, Single Point	*P*, Multipoint	LOD Score	*P*, Single Point	*P*, Multipoint	LOD Score
D17S1290/17q21.32/54.190	0.00114†	0.00142†	1.39*	0.00167†	0.00184†	1.34*	**0.00110†**	**0.00042†**	**2.63†**
D17S1301/17q21.33/55.759	**0.00003‡**	**0.000001‡**	**4.72‡**	**0.00003‡**	**0.000001‡**	**4.70‡**	**0.00013†**	**0.000001‡**	**6.76‡**
ACE/17q23/64.646	0.38275	0.03378	1.46*	0.36420	0.02958	1.57*	0.25981	0.01406	2.35†
D17S784/17q25.3/87.166	0.02866	0.01184	0.58	0.02781	0.01087	0.65	0.01924	0.00724*	1.10
ATA82B02/18q22/77.836	0.02074	0.02561	1.38*	0.01876	0.02456	1.35*	0.03310	0.02154	1.22*
VWFP1/22q11.21/15.543	0.00017†	0.00323*	0.87	0.00015†	0.00323*	0.94	0.00007‡	0.00088†	1.13

LDL-PPD1, LDL-PPD2, and LDL-PPD3 indicate LDL-PPD adjusted for (1) age, (2) age and BMI, and (3) age, BMI, and triglyceride, respectively. Results from single point and multipoint are reported in *P* values, and results from the variance components–based method are reported in LOD scores. Markers with highly suggestive evidence of linkage ($P \leq 0.0023$ or LOD score ≥ 1.75) for the three linkage methods for one of the LDL-PPD phenotypes are indicated in boldface type.

*$P \leq 0.01$ or LOD score ≥ 1.18.

†$P \leq 0.0023$ or LOD score ≥ 1.75.

‡$P \leq 0.0001$ or LOD score ≥ 3.00.

§Chromosome locations for marker with D number or marker with gene name are from the Location Database map,[40] and the other markers are from the Marshfield Institute map.[39]

LDL-PPD2 (LOD=4.70), and LDL-PPD3 (LOD=6.76). Marker D17S1290, located 1.6 cM from D17S1301, also gave fairly good evidence of linkage for the 3 phenotypes.

Other chromosomes exhibiting some evidence of linkage by the variance components–based method are displayed in Figure 1. Highly suggestive evidence of linkages was observed at 1p31 (leptin receptor locus), 2q33.2 (marker D2S1384), 4p15.2 (D4S2397), 5q12.3 (D5S1501), and 14q31.1 (D14S53). Markers at the leptin receptor locus and markers D5S1501 and D14S53 also provided evidence of linkage by the sib-pair method (see Table 2).

Other markers gave highly suggestive evidence of linkage ($P<0.0023$) with at least one of the linkage methods. For instance, marker D16S261 provided evidence of single-point linkage with the 3 phenotypes. The marker VWFP1 on chromosome 22q11.21 provided evidence of single-point and multipoint linkage for the 3 phenotypes. Conversely, marker D4S1627 yielded highly suggestive evidence of linkage for LDL-PPD1 and LDL-PPD2 with the variance component method. D5S1457 at 5p12 shows highly suggestive evidence of linkage in multipoint analysis for the 3 phenotypes and in single-point for LDL-PPD3. Finally, several markers provided highly suggestive evidence of linkage with LDL-PPD3 only, including D1S198, D2S434, IRS1 (2q36.3), ADRB2 (5q31), TNFα (6p21.3), D8S1110, D9S1121, D16S410, and ACE (17q23).

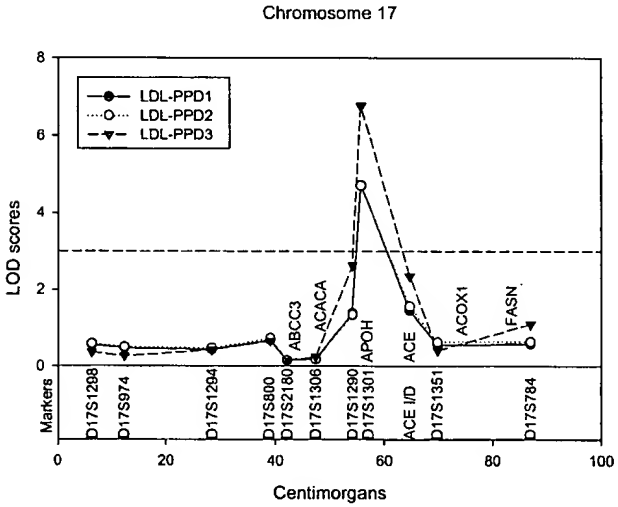

Figure 2. Quantitative transmission disequilibrium test linkage results for chromosome 17 with LDL-PPD phenotypes. Genetics markers used for linkage are indicated under *x* axis. Approximate location of candidate genes in vicinity of major peak are displayed on graph. Dashed horizontal line represents a LOD score of 3.00. LDL-PPD1, LDL-PPD2, and LDL-PPD3 indicate LDL-PPD adjusted for (1) age, (2) age and BMI, and (3) age, BMI, and triglyceride, respectively.

Discussion

The primary objective of this study was to identify QTLs affecting LDL-PPD variation. The results provide evidence for a major locus affecting LDL-PPD located on chromosome 17q21. Interestingly, none of the candidate genes located in the area of this QTL were tested previously. The marker D17S1301 located on chromosome 17q21.33 was strongly linked with the LDL-PPD, whether adjusted for covariates or not. However, the evidence for linkage was stronger when the phenotype was adjusted for plasma triglycerides, indicating that triglyceride levels may attenuate the penetrance of the locus. Marker D17S1290, located 1.6 cM from D17S1301, also provided good evidence of linkage ($1.34 \leq LOD \leq 2.63$). The apoH (APOH) gene, also referred to as β_2-glycoprotein I, is encoded under the peak linkage on 17q21. ApoH is a single-chain glycoprotein that exists in plasma both in a free form and in combination with lipoprotein particles. It has been implicated in several physiological pathways, including lipid metabolism, coagulation, and the production of antiphospholipid antibodies. This apolipoprotein activates lipoprotein lipase,[47] and genetic variations in this gene have been associated with variation in HDL cholesterol and triglyceride levels.[48–50] ACE is also located in this genomic region. This enzyme cleaves the final intravascular step,

TABLE 3. Candidate Genes Within Chromosomic Regions Linked to LDL-PPD

Chromosome Region	Marker	LOD Score LDL-PPD1	LDL-PPD2	LDL-PPD3	Candidate Genes
1p31	LERP	2.05	2.04	2.56	PLA2G2D, PLA2G2A, PLA2G5, APOER2, FABP3, CPT2, ABCD3, HMGCS2
2q33.2	D2S1384	1.91	2.11	2.27	PLA2R1, ABCB11, LRP2, ACADL, FACL3, ABCB6, IRS1, HDLBP
4p15.2	D4S2397	2.14	2.22	2.11	LRPAP1
5q12.3	D5S1501	2.40	2.34	2.10	HMGCR, HMGCS1
14q31.1	D14S53	2.79	2.65	1.72	ABCD4, CYP46
17q21.32-q21.33	D17S1290	1.39	1.34	2.63	ACACA, ABCC3, ACE, APOH, ACOX1, FASN
	D17S1301	4.72	4.70	6.76	

LDL-PPD1, LDL-PPD2, and LDL-PPD3 indicate LDL-PPD adjusted for (1) age, (2) age and BMI, and (3) age, BMI, and triglyceride, respectively.

resulting in the vasoactive peptide angiotensin II. Angiotensin II has been shown to bind specifically to LDL,[51] which produces a modified form of LDL that is taken up by macrophages at an enhanced rate, leading to cellular cholesterol accumulation.[52] In the present study, the insertion/deletion polymorphism in intron 16 of the ACE gene provided evidence of linkage with LDL-PPD1 (LOD=1.46), LDL-PPD2 (LOD=1.57) and LDL-PPD3 (LOD=2.35). Figure 2 shows the approximate location of candidate genes surrounding the major peak on chromosome 17.

Several other chromosomal regions provided highly suggestive ($P<0.0023$) evidence of linkage. These regions include chromosomes 1p31, 5p12-p12.3, and 14q31.1, which show evidence of linkage with both linkage methods and for all LDL-PPD phenotypes. Some promising candidate genes are located within these regions. First, the strongest evidence of linkage on chromosome 1p comes from a marker located within the leptin receptor (LEPR) gene. By modulating the hypothalamic effects of leptin on food intake and energy expenditure, genetic variants in the LEPR may affect energy balance and the size of LDL particles as a consequence of body fatness alterations. However, adjusting the LDL-PPD for BMI did not affect the strength of the linkage. On 1p, 3 members of the phospholipase A_2 (PLA2) gene family are present, namely PLA_2 group IID (PLA2G2D), group V (PLA2G5), and group IIA (PLA2G2A). PLA_2 is known to hydrolyze the phospholipid monolayers of LDL particles and change their physicochemical properties and size.[53] ApoE receptor 2 (APOER2) is also located near the locus of interest. On chromosome 5, 2 markers (D5S1457 and D5S1501) located 20 cM apart provided evidence of linkage with LDL-PPD. This region contains the HMG CoA reductase (HMGCR), which is the rate-limiting enzyme for cholesterol synthesis. A list of other potential candidate genes within the chromosomal regions linked to the LDL-PPD is provided in Table 3.

Among the panel of markers included in the genome scan, few candidate genes for LDL-PPD were present. First, an apoB marker gave no evidence of linkage with the phenotype. A significant linkage to apoB has been reported in a sib-pair linkage analysis of dizygotic female twins,[33] but other linkage studies excluded the hypothesis of linkage for the apoB locus and LDL size.[25,26,28] Second, although no linkage was found with the lipoprotein lipase locus in the present study or in 2 others,[28,34] a highly significant LOD score of 6.24 was obtained in another study of heterozygous lipoprotein lipase–deficient families.[30] Third, the apoE gene gave no evidence of linkage, as reported previously.[28,33,34] Finally, consistent with 3 other studies,[32–34] the LDL receptor also was not linked to LDL-PPD in the present study. In contrast, 2 previous results linked the LDL receptor locus to LDL subclass in families ascertained through probands with the atherogenic lipoprotein phenotype[27] and in families with coronary heart disease.[28] However, no amino acid sequence changes in the LDL receptor were found in the former study,[27] making it unlikely that a mutant allele in the LDL receptor gene was responsible for the linkage.[29] In the present study, negative results were also obtained with other candidate genes, including paraoxonase, hormone-sensitive lipase, CD36, and the intestinal fatty acid-binding protein.

In conclusion, the results of this study reveal the presence of a major locus located on chromosome 17q21.33 influencing LDL-PPD. This finding supports results from a handful of segregation analyses indicating the presence of a putative major locus for LDL particle size. Evidence of linkage was also found on chromosomes 1p31, 2q33.2, 4p15.2, 5q12.3, and 14q31.1. These QTLs harbor a good number of candidate genes that have not previously been tested in association studies with LDL-PPD.

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